Characterisation of Cellular and Viral Genes induced by the Adenovirus E1A gene

by

Anna Wanda Skulimowski

A thesis submitted for the degree of Doctor of Philosophy

of the Australian National University

March, 1990

STATEMENT

Unless stated below, work presented in this thesis was done by the candidate. Synthesis of the subtracted cDNA library, as described in Appendix 1, the initial differential screening of the library with cDNA probes,and screening of the selected clones with adenoviral probes and the thymidine kinase gene, as described in chapter 3 was done by Dr. E. Thalia David. Viral stocks were routinely maintained by Liz Bennett and Mary Silvestro. The electron microscopy experiment described in Chapter 4, was done in collaboration with Leslie Maxwell and Sue Bell of the Electron Microscopy Unit, John Curtin School of Medical Research.

A Skulimowski

Anna Skulimowski

ACKNOWLEDGEMENTS

This work was carried out during the tenure of a Commonwealth of Australia Postgraduate Research Award in the Department of Experimental Pathology/Division of Cell Biology at the John Curtin School of Medical Research.

I would like to thank Dr. Alan Bellett for his attentive supervision and his critical reading of this thesis. I am extremely grateful for the encouragement and stimulating discussions provided by my co-supervisor Dr. Ian Ramshaw. I also thank Ian for letting me have exclusive use of his (my?) computer while writing up. I would like to thank my advisors; Dr. Phil Board for informative comments about ubiquitin, Dr. Ken Reed for technical advice and Dr. Antony Braithwaite for being a mine of scientific information. I am indebted to Dr. Jeremy Weinman for his invaluable discussions on sequence analysis. I also benefitted from helpful discussions with Dr. George Miklos. For technical support and for creating an enjoyable enviroment to work in, I thank Jill Medveczky, Pam Bleakley and Margaret Maxted. I would to thank members of the Photography Unit for assistance in the preparation of figures in this thesis, and Andrea Morrison for secretarial assistance.

I am very fortunate to have the friendship and unending support of Drs. Sue and Scott Phillips (and Natalie). Thanks also to Dr. Janet Ruby for her encouragement and "sympathetic ear". I am very grateful for the support of my fellow students who were writing up at the same time, Cheryl, who could always outdo me in thesis horror stories and Guna, for his fabulous chicken curries and hot seafood soup. I thank Shaun for frequently uplifting my spirits. I am also grateful for my friends at University House, and for the Gang-gangs, Galahs, Crimson Rosellas and Sulphur-crested cockatoos that visited me every morning, for making life enjoyable.

I would like to thank all the members of the defunct Department of Experimental Pathology and more recently, the Division of Cell Biology, for helping to make this an interesting experience.

Finally, I thank Bill for his patience, understanding and ability to keep me sane, and my parents and my sister Barbara, for their endless faith, support and encouragement through the years.

ABBREVIATIONS

AAV2	Adeno-associated virus type 2
aa	amino acid
Amp	Ampicillin
BLOTTO	powdered milk, Diploma, dissolved in sterile water
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
dATP	Deoxy-adenosine-5'triphosphate
dCTP	Deoxy-cytidine-5'triphosphate
dGTP	Deoxy-guanosine-5'-triphosphate
dNTP	Deoxy-nucleotide triphosphates
dTTP	Deoxy-thymidine-5'-triphosphate
DTT	Dithiothreitol
dep	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol bis(ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid
FCS	Foetal calf serum.
g	Relative centrifugal field
iu	Infectious units
LB	Luria Broth
MOI	Multiplicity of infection
NP40	Nonidet P40
PBS	Phosphate buffered saline
PEI-cellulose	Polyethyleneimine-cellulose
pi	Post infection
poly A^+RNA	Polyadenylated RNA
REF	Rat embryo fibroblasts
RNA	Ribonucleic acid
RNAse	Ribonuclease, Promega
RT	Room temperature
S	Sedimentation coefficient
SDA	Selective DNA amplification
SDS	Sodium dodecyl sulphate

TE	10mM tris-HCl pH 7.5, 1mM EDTA
TEMED	Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
1U	Amount of enzyme required to digest 1µg DNA in 1hr at the manufacturer's recommended temperature
uv	Ultraviolet light
WT	Wild type adenovirus type 5
yeast tRNA	Transfer ribonucleic acid from bakers yeast

ABSTRACT

The ability of adenoviruses to transform primary cells in culture results from a culmination of many complex interactions between the cell and the regulatory regions of the virus to create an intracellular environment conducive for deregulation of cell growth, leading to immortality and transformation. Central to the process of transformation are the polypeptides transcribed from the E1A region of adenovirus. Alone, the products from the E1A region induce immortalization of semipermissive cells. In the presence of E1B, or a second oncogene, E1A products establish fully transformed cells. The functions of the E1A products include transactivating other viral genes, binding cell-cycle regulatory elements to induce the cell into S phase, and increasing the level of DNA synthesising machinery, by transactivating the expression of some genes, and repressing the expression of others. The full scope of cellular responses to E1A products has not been elucidated.

To identify genes, whose expression is induced by E1A products during the early stages of infection, and may be crucial in the establishment of a transformed phenotype, a subtracted cDNA library, enriched for E1A-induced sequences, was differentially screened. cDNA clones that reflected cellular sequences induced in WT infected cells and not in dl 312 (E1A negative) infected cells, were characterised by Northern analysis and sequencing, and were found not to play critical roles in transformation progression. A number of sequences, induced by WT infection, proved to be homologous to AAV sequences, indicating AAV contamination within adenovirus stocks. A repetitive sequence, related to the B2 family of repetitive sequences was also identified as an E1A-induced cellular sequence, providing direct evidence that adenovirus infection stimulates the transcription of an endogenous gene transcribed by RNA polymerase III.

Adenovirus infection was shown to increase expression of a polyubiquitin gene, Ub B. This could result in increased ubiquitin levels, leading to increased activity of an ATP dependent protein-degradation pathway, which could rapidly degrade foreign virion components, resulting in an inhibition of the lytic cycle. This mechanism could aid the establishment of a transformed state, although increased protein degradation by the ATP dependent pathway during adenovirus infection has yet to be confirmed.

TABLE OF CONTENTS

Chapter 1

Adenovirus Review

1.1. *Adenoviruses*

Adenoviruses were initially isolated from the lymphadenoid tissue of young children in 1953 by Rowe and his colleagues (Rowe *et al.* 1953) and independently isolated from military recruits suffering from acute respiratory illness by Hilleman and Werner in 1954 (Hilleman and Werner 1954). Over the next eight years many closely related viruses were isolated from other animal species including mice (Hartley and Rowe 1960), dogs (Kapsenberg 1959) and monkeys (Hull *et al.* 1958), leading to characterisation of the adenovirus group into 6 subgroups, based on natural host species (human, simian, bovine, canine, murine and avian) (Pereira *et al.* 1963). This thesis is entirely concerned with the human adenoviruses.

Adenoviruses are widespread amongst human populations. 41 different species have been recognised, assocaiated with a variety of diseases, ranging from acute respiratory illnesses to conjunctivitis and arthritis (Beladi 1972, Hilleman and Werner 1954, Rowe *et al.* 1956, Kibrick *et al.* 1957). Adenoviruses became scientifically attractive to laboratory investigators when Trentin and his colleagues (1962) found that adenovirus type 12 induced malignant tumours in hamsters when inoculated as newborns. This observation was extended to a number of other serotypes (Huebner *et al.* 1962, Huebner *et al.* 1965, Trentin *et al.* 1968) and to simian, canine and avian adenoviruses (Hull *et al.* 1965, Sarma *et al.* 1967, Sarma *et al.* 1965). The 41 different species of human adenovirus were subdivided into 5 groups, based on their oncogenicity and DNA homology as shown in table 1. Human adenoviruses became the focus of intensive research and were initially established as a model for the study of the mechanism of transformation and later, as a model to study eukaryotic gene expression.

Table 1.1 Characteristics of Human Adenoviruses [a]

Subgenus	Species	Oncogenicity[b]	DNA homology[c]	DNA GC (%)	Clincal manifestations
A	12,18,31	high	48-69%	47-49	Enteric infections
B	3,7,11,14,16, 21,34,35	weak	89-94%	49-52	Respiratory infections, urinary infections
C	1,2,5,6,	none	99-100%	57-59	Respiratory infections
D	8-10,13,15, 17,19,20,22-30,32,33,36	none	94-99%	57-59	Epidemic keratoconjunctivitis
E	4	none	NR[d]	57-59	conjunctivitis, respiratory infections
F[e]	40,41	NR	62-69%	ND[f]	acute gastroenteritis

[a] Modified from Green *et al* (1979), Gallimore *et al* (1985)

[b] Viruses and DNA of all species can transform rodent cells

[c] DNA homology within subgenus

[d] Not reported

[e] Data from van Loon *et al* (1985)

[f] Not done

1.2. *The Adenovirus genome*

While most genetic analysis has centered on the subgroup C viruses, in particular adenovirus types 2 and 5, the genomic organisation of the other serotypes appears to be similar. The adenovirus genome is a linear double stranded DNA molecule of molecular weight 20-25x10^6 daltons (Green *et al.* 1967). The DNA comprises of approximately 34-36kb (van Ormondt and Galibert 1984). There are inverted repeat sequences at the termini of the DNA, ranging in size from 100-160bp, depending on serotype (Garon *et al.* 1972, Arrand and Roberts 1979, Garon *et al.* 1982), and a protein of 55K molecular weight is covalently attached to the 5' end of both strands (Robinson *et al.* 1973, Rekosh *et al.* 1977).

The adenovirus genome is transcribed in both the rightward (from the r-strand) and leftward (from the l-strand) direction, as illustrated in the transcription map of adenovirus type 2 (figure 1.1). During productive infection adenovirus genes are transcribed in a complex order, and have been subdivided into 3 major groups. Early genes (transcribed in the order E1A, E4, E3, E1B and E2) are expressed before the onset of DNA replication. Intermediate genes (IX and IVa2) are expressed at low levels early in infection, increasing throughout infection even in the absence of DNA synthesis. Late genes are transcribed from the major late promoter (MLP) after the onset of adenovirus DNA replication.

1.3. *Early gene expression*

1.3.1. *The E1A region*

Transcription from the E1 region is detected first soon after infection (Nevins *et al.* 1979, Spector *et al.* 1978). The E1 region (1.3-11.2 mu) encompasses two genes - E1A and E1B, each encoding multiple mRNA species, which will be referred to by their sedimentation coefficients. The E1A gene is transcribed from the r-strand between 1.3 and 4.6 mu, producing 3 major mRNAs, a 13S (1.1kb), 12S (0.9kb) and 9S (0.6kb) mRNA. The 9S mRNA is transcribed late in infection (Berk and Sharp 1977, Spector *et al.* 1978, Kitchingman and Westphal 1980). Two species

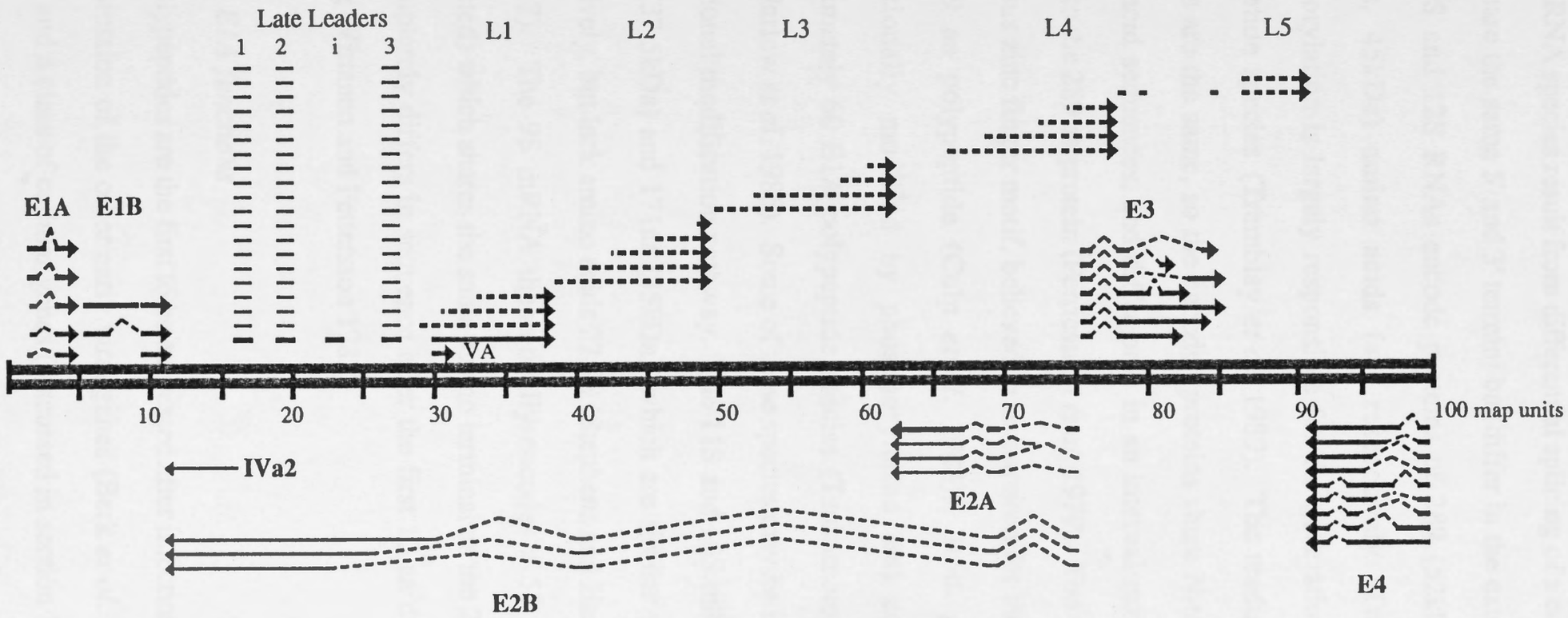


Figure 1.1 Transcriptional organization of the Ad 2 genome. The direction of transcription is indicated by arrows . Bold lines indicate transcripts detected early in infection, before DNA replication. Fine lines represent transcripts detected early and late in infection. The bold dashed lines represent transcripts detected late in infection. Adapted from Shenk and Williams 1984.

of mRNA, 10S and 11S mRNA are also detected, concomitant with 9S expression (Svensson *et al.* 1983, Stephens and Harlow 1987, Ulfendahl *et al.* 1987).

The mRNA species result from differential splicing of a common RNA precursor. They share the same 5' and 3' termini but differ in the extent of internal splicing. The 13S and 12S RNAs encode proteins of 289 (52kDa, 48.5kDa) and 243 (50kDa, 45kDa) amino acids (aa) respectively (Tremblay *et al.* 1989). Phosphorylation is largely responsible for the generation of the multiple E1A polypeptide species (Tremblay *et al.* 1989). The reading frames between the mRNAs are the same, so the encoded proteins share N-terminal and C-terminal amino acid sequences, and differ only in an internal region of 46 amino acids, unique to the 289 aa protein (Perricaudet *et al.* 1979). The 46 aa domain contains a consensus zinc finger motif, believed to be required for the correct functioning of the 289 aa polypeptide (Culp *et al.* 1988). Both polypeptides are post translationally modified by phosphorylation and acylation, resulting in approximately 60 E1A polypeptide species (Tsukamoto *et al.* 1986, Yee *et al.* 1983, Harlow *et al.* 1985). Some of these species may be intermediates in the post translational modification pathway. The 11S and 10S mRNA encode proteins of 217aa (37.5kDa) and 171aa (35kDa), which are similar to the 289aa and 243aa respectively, but lack amino acids 27-98 (Stephens and Harlow 1987, Ulfendahl *et al.* 1987). The 9S mRNA theoretically encodes a 55 aa polypeptide (as yet undetected) which shares the same amino terminal as the 289 and 243 aa proteins, but completely differs in sequence after the first 13 aa due to a frameshift after splicing (Virtanen and Pettersson 1983).

1.3.1.i. *E1A functions*

E1A polypeptides are the first to be expressed after infection and these transactivate the expression of the other early viral genes (Berk *et al.* 1979, Jones and Shenk 1979a) and a class of cellular genes (discussed in section 1.8.). E1A products are central to the process of oncogenic transformation by adenoviruses. In

collaboration with E1B or other viral oncogenes (discussed in section 1.6.) E1A products will transform rodent cells. In the absence of a second oncogene, E1A products are capable of immortalizing primary cells (Houweling *et al.* 1980, Ruley 1983). In addition to positive regulatory effects E1A proteins can repress transcription from certain viral and cellular enhancers. E1A proteins are found in the nucleus. The carboxy terminal sequence is necessary for localization (Lyons *et al.* 1987, Slavicek *et al.* 1989). A minority of the 289aa protein is associated with the nuclear matrix (Schmitt *et al.* 1987, Yee *et al.* 1983).

After comparison of the amino acid sequence of four different adenovirus serotypes, Ad4, Ad7, Ad2 and Ad12, three conserved regions within the E1A proteins were described by Tokunaga and his colleagues (1986). Conserved domain 1 (CD1) spanned from 41 to 63 aa, conserved domain 2 (CD2) ranged from 120-138 aa and conserved region 3 (CD3) was between 155 and 177 aa. CD1 and CD2 are contained within both the 243aa and 289 aa proteins, while CD3 is restricted to the unique region within the 289 aa protein. Moran and Mathews (1987) postulated that more broadly defined conserved regions (CD1 40-80 aa, CD2 120-139 aa, CD3 139-186 aa) were each responsible for different functions of the E1A proteins. It has been suggested that CD1 and CD2 are essential for transcriptional repression and transformation and CD3 is necessary for transcription activation. A wealth of evidence has been gathered to support these hypotheses, although evidence will be presented that suggests the E1A proteins are far more complex than originally imagined.

1.3.1.i.a *E1A mediated transcriptional repression*

In transient expression assays both E1A proteins were found to repress transcription from the SV40 early promoter (Velcich and Ziff 1985). The promoter regions of the muscle-specific α-skeletal and α-cardiac actin genes are also the site of E1A inhibition (Webster *et al.* 1988). Borrelli and his colleagues (1984) found that E1A products repress the enhancer activity of SV40, polyoma and E1A.

During infection of plasmocytoma cells, the E1A proteins repressed the enhancer of the immunoglobulin heavy chain (Hen *et al.* 1985). The E1A proteins inhibit enhancer-stimulated transcription of the rat insulin II gene. The same regions of the insulin enhancer are negatively regulated by cellular transcription factors (Stein and Ziff 1987, Stein and Whelan 1989). Repression of cytochrome P-450c gene expression, caused by blocking the enhancer element, has also been observed after cotransfection with E1A DNA in transient expression systems (Sogawa *et al.* 1989). Much interest has focused on the dissection of different functions (transformation, enhancer-mediated repression and transactivation) to different domains of the E1A proteins.

1.3.1.i.b *E1A-mediated transactivation and transformation*

The isolation of cDNA viruses, where the E1A region has been replaced by cDNA coding for the specific E1A proteins, has been particularly invaluable for functional analysis. Moran and her colleagues (1986a) constructed viruses containing 13S cDNA (13S virus), the 12S cDNA (12S virus) and the 9S cDNA (9S virus) in the place of the E1A region. It was demonstrated that the 13S virus grew as well as WT on Hela cells, while the 12S virus grew less well. The 9S virus was very defective for growth. The ability to transactivate expression of the E2 promoter was analysed by Northern and Western analysis, while E3 activation was monitored by a transient expression assay. The 12S and 9S viruses had lost the ability to transactivate the E2 and the E3 promoter (although the results show a 3 fold increase of expression from the E3 promoter by 9S). Although the 12S virus was defective for lytic growth, it immortalised baby rat kidney (BRK) cells at high efficiency. It was demonstrated that the 13S product could also immortalise BRK cells when transfected. The conclusion drawn from these experiments was that the 13S was necessary for transactivation and the transforming function was shared between the 13S and 12S products. Infection of BRK cells with the 12S virus resulted in the production of a growth factor which induced DNA synthesis and

proliferation of quiescent primary epithelial cells (Quinlan *et al.* 1987), suggesting that the 12S product could induce DNA synthesis.

The product of the 13S mRNA was also responsible for stimulating c-*fos* and c-*myc* promoter activities during a cotransfection assay in murine NIH 3T3 fibroblasts, while c-Ha-*ras* transcription was not affected (Sassone-Corsi and Borrelli 1987). The 12S mRNA product was found to have no effect (Sassone-Corsi and Borrelli 1987). In contrast, E1A products were found to be responsible for the reduction of c-*myc* in E1-transformed cell lines (Timmers *et al.* 1988).

The assignment of specific functions to defined conserved domains was similarly justified by the evidence obtained through analysis of the effects of mutations of E1A cDNA within plasmids in transfection assays by Montell *et al.* (1982), Lillie *et al.* (1986), Smith *et al.* (1986), Moran *et al.* (1986b), Kuppuswamy and Chinnadurai (1983), Lillie *et al.* (1987), Green *et al.* (1988) and Lillie and Green (1989). The relevance of the 3 functional domains within a biological system was recognised by evidence gathered from studies of viruses mutated within the conserved regions (Hurwitz and Chinnadurai 1985, Zerler *et al.* 1987, Whyte *et al.* 1988, Moran and Zerler 1988, Smith and Ziff 1988).

In recent years a significant body of evidence has accumulated that questions the emphasis placed on the existence of distinct domains of function within E1A. Winberg and Shenk (1984) constructed dl 347 and dl 348, containing a copy of 12S cDNA and 13S cDNA respectively, and found that both viruses grew efficiently, although not as efficiently as WT, in Hela cells. Both viruses were able to transactivate expression from the E2 and E4 promoters, although dl 348 was more efficient. The growth kinetics of dl 347 and the transactivation results implied that CD3 was not essential for transactivation.

Other evidence supporting the hypothesis that both E1A proteins are important in E1A associated functions include the observation that both proteins were required to cooperate with E1B to produce a transformed phenotype (Montell *et al.* 1984). Studies with plasmids containing mutated E1A genes showed that both 12S and 13S products could stimulate the rate of transcription from E2A and E3 promoters (Leff *et al.* 1984). Experiments involving microinjection of E1A protein products into Xenopus oocytes indicated that CD1 was sufficient for transactivation of the E3 promoter (Richter *et al.* 1985). Nakajima and his colleagues (1987) demonstrated that, when stably integrated into cell lines, the 13S and 12S products were each able to cause progression of the cell cycle at a similar rate. After intensive mutation analysis, Jelsma and his colleagues (1988, 1989) and Fahnestock and Lewis (1989) concluded that the E1A proteins contained different transactivation domains for different target genes, and sequences outside of the conserved domains influenced the outcome of transactivation, transformation and enhancer induced repression. Bellett *et al.* (1989) found that mutations within the E1A region affected all functions of E1A to different extents in biological assays of cell cycle progression, disruption of actin stress fibers and activation of viral gene expression.

The controversy about the location of functions within E1A proteins, as reviewed above, may be the result of the different target promoters in different assays. Perhaps the induction of different target promoters require different regions of the E1A proteins interacting with a broad range of transcription factors. E1A proteins do not transactivate transcription by binding to specific DNA sequences (Ferguson *et al.* 1985, Chatterjee *et al.* 1988) A number of investigators have suggested that E1A increases the activity of host cell transcription factors (Leong and Berk 1986, Kovesdi *et al.* 1986, Reichel *et al.* 1988). E1A products were found to enhance the transcription activity of RNA polymerase III, by increasing the concentration and phosphorylation of transcription factor IIIC (TFIIIC) (Yoshinaga *et al.* 1986, Hoefler *et al.* 1988).

The increase of expression of RNA polymerase II-transcribed genes by E1A is mediated by the activation or increase in concentration of a number of transcription factors, including activation transcription factor (ATF) and E2 promoter-binding factor (E2F) (Kovesdi *et al.* 1986, Lee *et al.* 1987, Kovesdi *et al.* 1987, Reichel *et al.* 1988). The ATF is identical to the transcriptional factor cAMP responsive element binding protein (CREB), which binds to a consensus sequence (CRE sequence), required for induction by cyclic AMP in several cellular promoters (Roesler *et al.* 1988). There are CRE sequences within all of the early gene 5'-flanking domains, including E1A. It is believed that E1A may interact with targets of the cAMP-signalling pathway to activate transcription of the other early genes (Sassone-Corsi 1988, Lin and Green 1988, Engel *et al.* 1988). Wu and her colleagues (1987) found that E1A transactivation of E1B is due to E1A modification of the cellular transcription factor TFIID (TATA-binding protein). By synthesising different transcription factor binding sites, to replace the E1B promoter in recombinant viruses, Pei and Berk (1989) demonstrated that the E1B TATA box, a CREB/ATF binding site and two E2F sites can mediate E1A transactivation. Thus E1A products stimulate transcription through multiple, distinct promoter elements.

1.3.1.ii. *Transactivation of DNA binding proteins by E1A*

As mentioned above, E1A products transactivate the expression of c-*fos* and c-*myc* protooncogenes during transfection studies (Sassone-Corsi and Borrelli 1987). An upstream element found at position -57 to -63 in the human, mouse and chicken c-*fos* genes resembles the consensus sequences of the CREB/ATF binding site (Montminy *et al.* 1986, Verma and Sassone-Corsi 1987). An upstream element between positions -95 to +47 in respect to the second promoter of the human myc gene is the target for adenovirus activation (Lipp *et al.* 1989).

Both fos and myc are nuclear proteins that participate in transcriptional regulation, and are induced in a variety of resting cells after stimulation with serum or growth factors (Setoyama *et al.* 1986, Muller *et al.* 1984, Kelly *et al.* 1983, Greenberg and Ziff 1984, Cochran *et al.* 1984, McNerney *et al.* 1987, Kruijer *et al.* 1984, Muller *et al.* 1985). Fos and myc have recently been recognised as DNA binding proteins which contain α-helical structures, known as leucine zippers (Landschulz *et al.* 1988). A leucine zipper is comprised of a sequence of 35 amino acids containing 4-5 leucine residues separated from each other by 6 amino acids, preceded by a region of positively charged amino acids. The periodicity arranges the leucine residues to one side of the amphipathic α helix. Leucines on two such helices would interdigitate, resulting in dimerization (Landschulz *et.al.* 1988). The dimerization would allow the regions of positively charged amino acids to assume a configuration that would specifically interact to a DNA recognition sequence. The fos protein alone does not dimerize, although it has DNA binding activity (Sambucetti and Curran 1986, Distal *et al.* 1987, Renz *et al.* 1987, Abate *et al.* 1990). Fos can form heterodimers with another transcription factor, jun (Kouzarides and Ziff 1988, Schuermann *et al.* 1989). The heterodimer shows increased affinity for the consensus binding site, AP-1, and increased transcription activation (Nakabeppu *et al.* 1988, Sassoni-Corsi *et al.* 1988, Chui *et al.* 1988). Dimerisation could stabilise a three dimensional protein structure, keeping the contact points for DNA binding in the correct orientation. As there are several different jun and fos-related proteins that complex in heterodimers, and recognise different DNA binding sites, there may be a diverse repertoire of cellular genes activated by these nuclear proteins, potentially inducible by E1A.

The presence of E1A is also essential for adeno-associated virus (AAV) replication. AAV is a defective parvovirus which requires the presence of a helper virus (usually adenovirus) for productive infection (Janik *et al.* 1981,Laughlin *et al.* 1982).

1.3.2. *The E1B region*

The E1B region (4.6-11.2 mu) is transcribed soon after E1A, E4 and E3 expression. The primary transcription product is spliced to 2 major mRNAs, 13S and 22S, and 2 minor species of 14S and 14.5S (Perricaudet *et al.* 1980, Virtanen and Pettersson 1985). In addition, an unspliced 9S mRNA encoding for the virion polypeptide IX, is transcribed from a separate promoter at intermediate and late times after infection (Alestrom *et al.* 1980). Each E1B mRNA contains 2 open reading frames (ORFs). The first open reading frame is contained within the first exon of the 13S, 14S, 14.5S and 22S mRNAs, resulting in a 175 aa (19kDa) polypeptide (Bos *et al.* 1981). The second ORF in the 22S RNA codes for a polypeptide of 495 aa (55kDa) (Ross *et al.* 1980). In the 13S, 14S and 14.5S mRNAs, the second ORF encodes polypeptides of 82 aa, 155 aa and 92 aa, respectively, which are translated in an altered frame due to differential splicing (Anderson *et al.* 1984).

1.3.2.i. *E1B protein functions*

Although E1A gene products play a predominant role in transformation as described above, induction of a full transformed phenotype and correct regulation of gene expression requires the expression of the E1B gene (Chinnadurai 1983, Barker and Berk 1987). E1B products have been shown to increase the rate of transcription-initiation of the E1A region in transformed primary cells (Jochemsen *et al.* 1987). The exact mechanisms of function of the two major E1B proteins has not been elucidated, however mutation analysis has allowed assignment of various activities to each protein. In some adenovirus-transformed cells, the 495aa protein is complexed with the cellular proto-oncogene p53, which was shown to localize to a filamentous body in the cytoplasm, thus sequestering p53 from the nucleus, and possibly through p53 inactivation, predisposing the cells to transformation (Sarnow *et al.* 1982, Zantema *et al.* 1985). Other investigators have shown that in most Ad2 and Ad5 transformed rodent cell lines the 495aa protein also complexed with p53 in discrete areas in the nucleus (Blair-Zajdel and Blair 1988). In productively infected

HeLa cells, which do not express the p53 protein, the 495aa protein is physically associated with an E4 34 kDa protein (Sarnow *et al.* 1984). In infected rodent cells, association of E1B with p53 occurs in the presence or absence of E4 (Braithwaite *et al.* 1990 submitted). In the absence of the 495aa E1B product, viral growth was slightly defective and transformation of rat cells was inhibited. The 495aa product was necessary for optimum transport and accumulation of viral late mRNAs and reduction of host cellular mRNA (Pilder *et al.* 1986) The 495aa product also facilitates the rescue of AAV from a latent state and the cytoplasmic accumulation of AAV mRNA in the cytoplasm (Samulski and Shenk 1988).

In the absence of the 175aa protein, host cell and viral DNA are extensively degraded late in infection, the onset of cytopathic effect is dramatically accelerated and the viruses are severely defective for transformation (Pilder *et al.* 1984, White *et al.* 1988). The correct localisation of the 175 aa protein to the nuclear envelope prevented the degradation of host DNA, suggesting that the E1B protein was a deterrent to the possible entry of cytoplasmic nucleases into the infected cell nucleus (White *et al.* 1984). Evidence suggests that the 175 aa protein is a negative regulator of E1A-dependant viral early gene transcription. In the presence of E1A, the 175aa product repressed E1A-dependent gene expression, in the absence of E1A, the E1B product was shown to increase viral gene expression and DNA synthesis (White *et al.* 1988). During transient expression assays, the 175 aa product physically interacted with, and disrupted, the organisation of intermediate filaments and the nuclear lamina, without affecting the other cytoskeletal networks (White and Cipriani 1989). The disruption of the intermediate filaments does not result in gross alterations in cell morphology, however by modifying cell morphology the occurrence of a cytopathic phenotype may be prevented. The nuclear lamina is essential for the maintenance of nuclear chromatin structure. By altering this structure the 175 aa protein could alter gene expression, or growth regulation, and may prevent DNA degradation, thus promoting transformation.

1.3.3. *The E2 region*

E2A (61.7-75 mu) and E2B (11.3-30.0 mu) are the two genes transcribed from the l-strand within the E2 transcription unit (Galos *et al.* 1979). Early in infection transcription initiates from a promoter at 72.5 mu, and transcription initiates at a different promoter (at 72mu) during late infection (Chow *et al.* 1979). Multiple mRNA species are generated from differential splicing of a primary transcript (Berk and Sharp 1977, Chow *et al.* 1979, Kitchingman *et al.* 1977). The E2 region encodes three proteins which are essential for adenovirus replication. The E2B unit codes for an 87kDa protein that is the precursor of the 55kDa terminal protein covalently bound to the 5'ends of adenovirus DNA. The 87kDa protein acts as a primer for DNA synthesis (Stillman *et al.* 1981). E2B also encodes a 140kDa DNA polymerase which is required for chain elongation during DNA synthesis at the origin of replication (Lichy *et al.* 1982). The E2A region encodes a single stranded DNA binding protein (DBP), which is synthesised both early and late during infection (Linne *et al.*, 1977).

1.3.3.i. *E2 functions*

The 72kDa (529 aa) DBP is critical for DNA replication. It is required for initiation and strand elongation during viral DNA synthesis (van der Vliet and Sussenbach 1975, Friefeld *et al.* 1983). Many other functions have been assigned to the DBP. DBP regulates the expression of viral late gene expression. Wild type (WT) adenovirus does not productively infect monkey cells due to reductions in the transcription rate of late genes, alterations in mRNA splicing patterns and poor utilization of the mRNA *in vivo* (Johnston *et al.* 1985, Anderson and Klessig 1984, Anderson and Klessig 1983). Mutant viruses which contain alterations in the DBP can overcome these block and productively replicate in monkey cells (Klessig and Grodzicker 1979).

The DBP was also believed to regulate the expression of itself and the other viral early genes. Carter and Blanton (1978) compared the relative abundance of early

viral RNAs between the WT virus and a temperature sensitive mutant virus, defective in the DBP at restrictive temperatures (Ad5ts125). They noted a 3-7 fold accumulation of early RNA, at restrictive temperatures during mutant infection, suggestive of negative regulation mediated by the DBP. The negative regulation was thought to occur post transcriptionally, at cytoplasmic RNA stability, for E1A and E1B (Babich and Nevins 1981), and affect the rate of transcription of E4 (Nevins and Jensen-Winkler 1980).

More evidence of eukaryotic translational control by DBP was presented by Jay and his colleagues (1981), who investigated the effect of coinfection with the DBP-defective mutant virus on the expression of structural proteins of adeno-associated virus (AAV). Accumulation of AAV capsid protein decreased significantly, on coinfection with Ad5ts125, although mRNA did not decrease and could be translated *in vitro*. It was concluded that control of translation of AAV structural proteins was influenced by the DBP.

The isolation of mutants with deletions in the DBP by Rice and Klessig (1985) made study of infection without the presence of DBP possible. Through the study of deletion mutants, no evidence was found of any negative or positive role of DBP in the regulation of the other adenovirus early genes (Klessig *et al.* 1986). DBP was shown to be associated with RNA *in vivo* and *in vitro*, implying involvement in viral RNA transcription and processing (Klessig and Grodzicker 1979, Cleghon and Klessig 1986, Klessig *et al.* 1986).

Both E2A and E2B contribute, perhaps indirectly, to the events leading to transformation by adenoviruses. Alterations in the E2A DBP enhanced transformation frequencies, due most likely to an activity possessed by the altered DBP, as viruses with total deletions of DBP transformed rat cells at frequencies similar to wild-type (Logan *et al.* 1981, Rice *et al.* 1987). Viruses containing mutations that map to E2B, particularly to the DNA polymerase, are defective for

DNA replication in human cells and transformation of rat cells (Miller and Williams 1987).

1.3.4. *The E3 region*

The E3 transcription unit (76.6-86 mu on the r-strand) produces approximately 16 mRNAs through differential splicing, which contain nine different open reading frames (Cladaras and Wold 1985, Cladaras *et al.* 1985, Brady and Wold 1988). E3 has been shown to be dispensable for the growth of virus in tissue culture (Kelly and Lewis 1973), and is thought to be involved in the protection of adenovirus from host defense mechanisms during natural infection. Seven polypeptides have been isolated, including a 19kDa glycoprotein (gp19kDa), the 11.6kDa, 10.4kDa and 14.7kDa proteins (Persson *et al.* 1980, Wold *et al.* 1985, Wold *et al.* 1984, Tollefson and Wold 1988).

1.3.4.i. *E3 functions*

The gp19kDa, a transmembrane protein, binds to certain class 1 major histocompatibility complex (MHC) antigens in the endoplasmic reticulum and prevents their transport to the cell surface, thus diminishing class 1 MHC expression on the cell surface (Andersson *et al.* 1985, Burgert and Kvist 1985, Paabo*et al.* 1987, Paabo *et al.* 1986). The 14.7 kDa protein inhibits cytolysis of infected cells in culture by tumor necrosis factor (Gooding *et al.* 1988, Wang *et al.* 1988). The 10.4KDa protein, a plasma-membrane associated protein, induces the endosome-specific internalisation of cell surface-associated epidermal growth factor receptor (EGF-R) and its subsequent degradation in lysosomes (Carlin *et al.* 1989, Tollefson *et al.* 1990). The function of the 11.6kDa is still uncertain.

1.3.5. *The E4 region*

The E4 region lies between 91.3 and 99.0 and is transcribed from the l-strand. DNA sequence and mRNA analysis have identified 7 open reading frames within 12-24 unique mRNAs, with the potential to express at least seven distinct protein

products (Virtanen *et al.* 1984, Fryer *et al.* 1984, Tigges and Raskas 1984). By *in vitro* translation of the mRNA of E4 and tryptic peptide map analysis, Matsuo and his colleagues (1982) identified polypeptides of 35kDa, 23kDa, 22kDa, 21kDa, 18kDa and 11kDa, although others have identified 16 polypeptides through similar techniques (Tigges and Raskas 1982). Three E4 proteins have been identified immunologically. A 34kDa protein from ORF 6 has been shown to be physically associated with the E1B-encoded 55kDa protein and thought to be involved with the transport of viral late mRNA from the nucleus to the cytoplasm (Sarnow *et al.* 1984, Cutt *et al.* 1987, Pilder *et al.* 1986). An 11kDa protein from ORF 3 is found associated with the nuclear matrix (Sarnow *et al.* 1982). A 19 kDa polypeptide was identified that maps to ORF 6/7 (Cutt *et al.* 1987). Through the construction and analysis of mutants which have deletions in specific ORFs it has been possible to assign roles to specific ORF products. The polypeptides from ORF 3 and ORF 6 can individually permit normal late viral mRNA accumulation and protein synthesis (Halbert *et al.* 1985, Bridge and Ketner 1989). In mutant-E4 infected cells there is a reduction in the stability of viral late RNAs in the nuclei, leading to a reduction of accumulation of viral late mRNA in the cytoplasm (Sandler and Ketner 1989). Adenovirus mutants which contain major deletions of E4 were also defective for virus growth at the level of DNA replication and assembly of virus particles as well as delayed shutoff of host cell metabolism (Halbert *et al.* 1985, Falgout and Ketner 1987, Weinberg and Ketner 1986). Recently E4 proteins have been shown to transactivate expression from the E2 promoter, by activation of the transcription factor E2F, in collaboration with E1A (Reichel *et al.* 1989, Babiss 1989).

1.4. *Intermediate gene expression*

Transcription from intermediate genes begins early in infection, at a low rate and increases substantially late in infection, even in the absence of adenovirus replication (Lewis and Mathews 1980). Two intermediate genes have been described. The IVa_2 gene maps at 11.3 to 16.1 mu and is transcribed from the l-strand (Chow and Broker 1978). The IVa_2 gene encodes for a 50kDa polypeptide,

believed to be a maturation protein during morphogenesis of the virion particle (Persson *et al.* 1979). From the E1B transcription unit, an unspliced 9S mRNA encoding for the virion polypeptide IX, is transcribed at intermediate and late times after infection (Alestrom *et al.* 1980). Several open reading frames have been identified in the region between 11.6-34 mu, although no known protein or function has been assigned to them. By treatment of cells with with protein inhibitors and DNA synthesis inhibitors Lewis and Mathews (1980) found an intermediate mRNA species of 13.5kDa which mapped to 17-21.5 mu. Two polypeptides of 16.5kDa and 17.0kDa have been mapped to 11.6 and 17.0 mu (Lewis *et al.* 1979).

1.4.1. *Virus associated RNAs*

Virus associated RNAs (VA RNA) are transcribed by RNA polymerase III from two genes (VAI and VAII) at 28.8 and 29.5 mu (Mathews 1975, Weinmann *et al.* 1976). The VAI and VAII are both approximately 160 nucleotides in length and are detectable early in infection, although VAI RNA is produced in large amounts late in infection. The VA RNAs are capable of extensive intramolecular base pairing and have been shown to anneal with viral mRNA (Akusjarvi *et al.* 1980, Mathews 1980). A small proportion of VA RNA is found in ribonucleoprotein particles in association with the La antigen (a cellular protein which binds to short runs of 3' terminal U residues) (Lerner *et al.* 1981, Francoeur *et al.* 1982, Stefano 1984).

Analysis of mutant viruses that were defective in the production of either one of the VA RNAs revealed that the absence of the VAI resulted in poor virus growth (Thimmappaya *et al.* 1982). In cells infected with the VAI mutant, protein synthesis is reduced by over 90% at late times of infection. It was concluded that the VAI RNA was required for efficient translation of late viral mRNAs. The VAI RNA was necessary for the efficient synthesis of AAV structural proteins (Janik *et al.* 1989). Further study with the VAI defective virus revealed that the VAI RNA exerted its effect during the initiation of translation (Reichel *et al.* 1985).

It was thought that VAI RNA was necessary to facilitate interaction between 43S preinitiation complex and mRNA to form the 48S species that would bind the 60S ribosomal subunit to yield an 80S initiation complex (Schneider *et al.* 1984). Polypeptide chain initiation requires the covalent modification of initiation factor eIF-2 to form eIF-2.GTP which then forms the ternary complex, eIF.GTP.Met-$tRNA_f$, which supplies the ribosome with the initiator amino acid. In the presence of guanosine nucleotide exchange factor (GEF) the eIF2 is catalytically recycled. As an anti-viral response the cell produces interferon, which in turn induces synthesis of a protein kinase, DAI (the double-stranded (ds) RNA activated inhibitor). The DAI phosphorylates eIF-2, preventing recycling and inhibiting chain elongation (Schneider and Shenk 1987, Ochoa 1983). VA RNA binds to DAI, thus preventing phosphorylation of eIF-2 (Katze *et al.* 1987, Mellitis and Mathews 1988, O'Malley *et al.* 1989). It was noted that eIF-2 phosphorylation increased during Ad 2 infection, correlating with the shut off of host protein synthesis. It is thought that there may be segregation of host and viral RNAs into different translational compartments. The viral compartment would contain viral mRNAs and VAI RNA, which would prevent DAI activation and thus permit active protein synthesis. The host mRNA compartment would not contain VAI RNA, thus DAI would be active in phosphorylating eIF-2 and inhibiting host protein synthesis (O'Malley *et al.* 1989).

1.5. *Late gene expression*

Upon replication of the adenovirus genome, transcription of late genes from the major late promoter (MLP) commences (Thomas and Mathews 1980). The late genes encode mainly structural proteins. The primary transcript extends from 16.45 to 99 mu on the r-strand (Goldberg *et al.* 1978, Ziff and Evans 1978, Weber *et al.* 1977, Fraser *et al.* 1979) and is differentially spliced to yield 15-20 mRNAs which are subdivided to 5 families, L1 to L5, on the basis of different polyadenylation sites at 38.5 mu, 63 mu, 79.5 mu and 91.5, respectively (McGrogan and Raskas 1978, Nevins and Darnell 1978, Fraser and Ziff 1978).

The mRNAs share a common tripartite 5' leader sequence of 3 short segments initiating at 16.45 mu, 19 mu and 27 mu (Berget *et al.* 1977, Chow *et al.* 1977).

The major late promoter is also expressed early in infection and its transcripts yield mRNA from the L1 family, although transcription does not proceed past 39 mu (Chow *et al.* 1979, Thomas and Mathews 1980, Shaw and Ziff 1980). The L1 mRNAs encode for two structurally related proteins of 52kDa and 55kDa which are found in the nucleus, associated with the nuclear membrane and persist throughout infection (Miller *et al.* 1980, Lucher *et al.* 1986). An i-leader sequence mRNA is also transcribed from the L1 region, which translates into a 16kDa protein found in the cytoplasm (Lewis *et al.* 1985, Symington *et al.* 1986). There are three other late proteins that are non structural. The 23kDa polypeptide encoded by L3 is believed to be a protease which cleaves the polypeptide precursors of the virion (Yeh-Kei *et al.* 1983). A 100kDa protein, produced from L4, is believed to function in hexon capsomer assembly and transport (Gambke and Deppert 1983). A 33kDa (39kDa) protein is encoded by L4, however it's function is unknown due to a lack of appropriate virus mutants (Oosterom-dragon and Anderson 1983). The structural proteins encode by the late region are II (L3, hexon), III (L2, penton base), IV (L5, fiber), V (L2, core), and precursors to VII (L2, core) and VIII (L4, hexon-associated) (Pettersson, 1984).

1.6. Adenovirus transformation

The E1 region was initially implicated as important in transformation when Graham and his colleagues (1974) demonstrated that adenovirus DNA sheared to fragments of less than 10% of the genome retained the ability to transform primary rat kidney cells. By using specific exonucleases, the transforming activity was located to 0-6% from the left terminal of the genome. Analysis of adenovirus-transformed rat cell lines revealed that all cell lines contained at least the left-terminal 14% of the viral DNA (Gallimore *et al.* 1974, Sambrook *et al.* 1975), suggesting that retention

of the genes within the left 14% of the genome was required for transformation, or maintenance of the transformed state.

Transformation of primary cells requires at least two functions, immortalization and transformation, encoded by two separate genes, E1A and E1B, in the leftward transforming region. Rat cells transformed by the E1A region possess an immortalized phenotype, believed to be the first stage of transformation. The cells are fibroblastic in morphology, are unable to grow to high saturation densities, are not tumourigenic, but have the potential for indefinite replication in media with serum (Shiraki *et al.* 1979, Houweling *et al.* 1980, Senear and Lewis 1986). Transformation of primary cells with E1A and E1B result in an oncogenic phenotype, characterised by anchorage-independent cell growth and the ability to form tumours in syngeneic animals (van den Elsen *et al.* 1982). The expression of E1B, without E1A, does not alter cell replication or morphology, and thus is not sufficient for morphological transformation (Solnick 1981, Solnick and Anderson 1982, van den Elsen *et al.* 1983). Therefore, E1A is central to oncogenic transformation.

E1A is a member of a class of nuclear oncogenes, that can cooperate with c-Ha-*ras*1to completely transform primary rat embryo cells and induce DNA synthesis in growth arrested cells (Ruley 1983, Braithwaite *et al.* 1983, Stabel *et al.* 1985, Kaczmarek *et al.* 1986a). These characteristics are shared with the polyoma and SV40 virus large T antigens, *myc*, N-*myc* and L-*myc* (Ruley *et al.* 1983, Land *et al.* 1983, Yancopoulos *et al.* 1985, Birrer *et al.* 1988). The mechanism of transformation by the adenovirus E1 region is not well understood. Yee and Branton (1985) and Harlow and his colleagues (1986) found that the 13S and 12S E1A products were able to form stable protein complexes with cellular polypeptides of molecular weights 28, 40, 50, 60, 80, 80, 105, 107, 130 and 300 kDa. The 60kDa polypeptide has recently been found associated with the mammalian homolog of yeast cdc2 protein kinase, believed to play a distinct role in the

regulation of the cell cycle (Giordano *et al.* 1989). Any mutations within the E1A proteins which destroyed the binding of E1A proteins to the most predominant cellular polypeptides of 300, 107 or 105kDa also inactivated the ability of E1A proteins to cooperate with a *ras* gene to transform baby rat kidney cells (Whyte *et al.* 1988). When the regions of E1A that bound the 300, 107 and 105kDa were more precisely mapped, the regions of E1A that are required for the interactions with the cellular polypeptides coincided with the regions of E1A that are required for its transformation function, ie CD2 (Whyte *et al.* 1989). The 105 and 107kDa proteins required overlapping regions on E1A proteins and associated in a mutually exclusive manner. The results suggest that the transforming activities of the E1A proteins depend on their stable interactions with specific cellular polypeptides. The cellular 105kDa polypeptide has been identified as the product of the retinoblastoma gene (Whyte *et al.* 1988).

1.7. *E1A interactions with cell cycle regulatory elements in infection and transformation*

1.7.1. *p60 associates with p34 protein kinase*

The 60kDa polypeptide that associates with the E1A proteins in virally transformed or infected cells, has recently been shown to complex with the mammalian homolog of yeast cdc2 protein kinase (Giordano *et al.* 1989). The 34kDa protein product (p34) of the cdc2 gene is the catalytic subunit of a protein kinase, localised in the nucleus, which is involved in cell cycle regulation in a wide number of eukaryotes (Riabowol *et al.* 1989, Simanis and Nurse 1986, Draetta and Beach 1988). In Xenopus, cdc2 has been identified as a component of maturation promoting factor (MPF), which causes an interphase cell to enter mitosis (Dunphy *et al.* 1988). In mammalian cells, the injection of anti-p34 antibodies blocked cell division without affecting DNA synthesis, suggesting that p34 acts in the initiation of mitosis (Riabowol *et al.* 1989).

There is rapid induction of p34 synthesis during infection, and consequent proliferation, of baby rat kidney (BRK) cells by adenovirus. BRK cells are induced to proliferate by adenovirus E1A (Montell *et al.* 1984, Quinlan and Grodzicker 1987). The induction of p34 synthesis is dependent on E1A expression, and does not require DNA synthesis (Draetta *et al.* 1988). The significance of the induction of p34, the association with p60 and the association of E1A proteins with p60 is not well understood.

In highly mitogenically activated HeLa cells, the cdc2-p34 protein interacts with a number of proteins. The complex of p34-p60 display a cell-cycle dependent histone 1 kinase activity which is most active in interphase (Giordano *et al.* 1989). A 13kDa suc1-related polypeptide acts as a subunit of the cdc2 protein kinase (Draetta *et al.* 1987). p34 also binds to a 62kDa polypeptide, which has been identified as human cyclin B. Mitotic cyclin increases during G2 and is abruptly degraded at mitosis to allow entry into interphase (Pines and Hunter 1989). It is thought that cyclin B is the major endogenous substrate of the cdc2 kinase complex. The cdc2-cyclin complex also acts as a histone H1 kinase, but is most active in mitotic metaphase (Giordano *et al.* 1989). The E1A proteins do not sequester all of the cellular pool of p60, as the properties of the cdc2-p60 complex do not change in virally infected cells. Little is known about the E1A-p60 complex. Perhaps the complex is responsible for the chromosomal aberrations and abnormal segregations that occur during mitosis of virus infected cells (Murray *et al.* 1982).

1.7.2. *The retinoblastoma susceptibility gene product*

Mutations in the retinoblastoma susceptibility gene (RB gene) in one allele results in a predisposition to retinoblastomas and osteosarcomas, while the loss of both alleles results in tumourgenesis (Godbout *et al.* 1983). Changes in the RB gene were also detected in human breast tumours, in small lung cell cancers and in a bladder carcinoma line (Lee *et al.* 1988, Harbour *et al.* 1988,Yokota *et al.* 1988, Horowitz *et al.* 1989). The RB gene contains 27 exons distributed over 200 kb and

encodes a mRNA of 4.7kb (Friend *et al.* 1986, Lee *et al.* 1987a, T'Ang *et al.* 1989). The RB gene encodes a nuclear phosphoprotein of approximately 110-114kDa that has DNA binding activity (Lee *et al.* 1987b). The RB protein ($p110^{RB}$) is expressed in all of the human tissues as yet analyzed, for example skin, skeletal muscle, kidney, retina, brain, and lung (Grand *et al.* 1989). Proteins antigenically related to the RB protein, and DNA sequences homologous to the RB cDNA have been identified in other vertebrate species eg. rats, mice, cats, chickens and cattle (Lee *et al.* 1987a,b).

The RB protein has an important role in constraining uncontrolled growth of cells. When the RB gene was introduced into RB^- tumour cells, via retrovirus-mediated gene transfer, the neoplastic phenotype, characterised by a rounded cell morphology, increased growth rate, soft agar colony formation and tumourgenicity in nude mice, was suppressed, suggesting that the Rb gene is a tumour supressor gene (Huang *et al.* 1988). Major viral products from DNA tumour viruses form specific complexes with the RB protein. The SV40 large T antigen has been shown to bind preferentially to an unphosphorylated member ($p110^{RB}$) of the RB family in virus-infected and -transformed cells (DeCaprio *et al.* 1988, Ludlow *et al.* 1989). The E7 oncoprotein of the human papilloma virus type 16 was found to complex with $p110^{RB}$ (Dyson *et al.* 1989b). The adenovirus E1A proteins bind both the unphosphorylated and phosphorylated forms of the RB proteins (Dyson *et al.* 1989b, Buchkovich *et al.* 1989). $p110^{RB}$ may be a common target for transformation by the DNA viruses.

A putative function was assigned to the RB products after the analysis of cell cycle-dependent phosphorylation of RB. $p110^{RB}$ is synthesised throughout the cell cycle, but is phosphorylated in a cycle specific manner. In synchronised cells at G0/G1 all the RB protein is unphosphorylated ($p110^{RB}$). RB phosphorylation (polypeptides $pp112\text{-}114^{RB}$), and the inactivation of the protein, occurs at the G1/S boundary and in G2 (DeCaprio *et al.* 1988, Buchkovich *et al.* 1989). $p110^{RB}$ is

believed to act as an active block to exit the G1 phase . Evidence to support this hypothesis includes the data that the SV40 T antigen bound unphosphorylated $p110^{RB}$ and the SV40 T antigen is a strong potent mitogen, able to drive G0/G1 cells into S (Soprano *et al.* 1981). This suggests that by binding $p110^{RB}$, thereby inactivating the protein, the SV40 T antigen removes the block from G1 progression into S, and by entering the S phase, the cells provide cellular factors required for viral replication (DeCaprio *et al.* 1988, Buchkovich *et al.* 1989).

The E1A polypeptides are capable of associating with the RB protein throughout all stages of the cell-cycle (Buchkovich *et al.* 1989). The interpretation of this result may be that the E1A proteins are targeting other activities of the RB protein in addition to its G1 activity. While binding to the $p110^{RB}$ by E1A products is essential for transformation, it is not sufficient for transformation. Mutant viruses that were able to bind $p110^{RB}$, but were unable to bind the cellular 300 and 107kDa proteins, were transformation negative (Egan *et al.* 1989). The cellular 107kDa nuclear phosphoprotein was also found to associate with the large T-antigens of SV40 and human polyoma virus (JC). Association with the 107kDa protein may represent another common component in transformation by E1A and large T antigen (Dyson *et al.* 1989a).

1.7.3. *p53*

E1B proteins are required in collaboration with E1A proteins to fully transform cells. The E1B proteins associate with a nuclear cell cycle regulatory element, namely p53, localizing it to a filamentous body in the cytoplasm, thus sequestering p53 from the nucleus, and possibly predisposing the cells to transformation (Sarnow *et al.* 1982, Zantema *et al.* 1985). In most Ad2 and Ad5 transformed rodent cell lines, however, the 495aa protein also complexes with p53 in discrete areas in the nucleus (Blair-Zajdel and Blair 1988). In chemically-induced transformed embryonic murine cells, p53 was concentrated in the nucleus, associated with chromatin, and only a minor fraction was detected in the cytoplasm.

p53 accumulation in the nucleus was also a feature of other carcinogen-treated primary fibroblasts. In the non-transformed parental cells, p53 was only detected in the cytoplasm (Rotter *et al.* 1983). p53-SV40 complexes were located on the network of nucleoplasmic fibrils of SV40-transformed cells, although p53 was associated with the plasma membrane of transformed and untransformed cells during mitosis (de Fromentel *et al.* 1986, Milner and Cook 1986). In normal peripheral blood lymphocytes, p53 was localised exclusively to the nucleus (Mercer and Baserga 1985). The differences in location may be due to the type of cell analysed.

p53 was initially identified as a cellular polypeptide that complexed with the SV40 T antigen in SV40-transformed cells (Lane and Crawford 1979, Linzer and Levine 1979). In tumours and transformed cell lines p53 was often found in increased levels, directly attributed to increased stability of the protein (DeLeo *et al.* 1979, Crawford *et al.* 1981, Oren *et al.* 1981, Reich *et al.* 1983, Rogel *et al.* 1985). p53 was shown to form high molecular weight hetero-oligomers and homo-oligomers in transformed cells, while p53 from normal and immortalised cells was found in low molecular weight forms (Kraiss *et al.* 1988). In transformed cells, p53 was found complexed to members of the 70kDa heatshock family (Hinds *et al.* 1987, Pinhasi-Kimhi *et al.* 1986, Sturzbecher *et al.* 1987, Finlay *et al.* 1988). It was subsequently cloned in a number of different laboratories from a SV40 transformed mouse cell line, a chemically transformed mouse cell line and a murine B cell lymphoma and shown to cooperate with activated *ras* in cellular transformation (Oren and Levine 1983, Jenkins *et al.* 1984, Eliyahu *et al.* 1984, Parada *et al.* 1984).

An immortalizing function was assigned to p53 when transfection of plasmids containing murine p53 sequences, under the control of the efficient Rous Sarcoma Virus long terminal repeat (RSV LTR), into adult chondrocytes (of finite lifespan) resulted in cellular immortality and potential for transformation by activated *ras*

(Jenkins *et al.* 1984). It was found that immortalization was conditional upon the promoter/enhancer construct used, since, under the control of the relatively weak SV40 early enhancer, p53 did not immortalize cells, unless alterations in the p53 coding sequence had occurred, resulting in stable p53 mutants. Thus transformation was dependent on the amount of available p53 protein. Immortalization of chondrocytes was achieved when the p53 cDNA construct contained the SV40 origin of replication, the early region promoter and enhancer (Jenkins *et al.* 1985).

Further evidence for the ability of the p53 protein to immortalize rodent cells was presented by Rovinski and Benchimol (1988). While comparing the immortalization potential between intact (53kDa) and rearranged (44kDa) p53 genes from Friend virus induced erythroleukemic cell lines and the p53 gene from a normal murine liver, they observed that the the p53 gene from the normal liver generated immortal cells with a high efficiency. All three genes, when placed into pUC18 with their natural promoters, immortalized early passage rat embryo fibroblasts after transfection. The rearranged p53 gene from a Friend virus induced erythroleukemic cell line (DP15-2), contained a 3kb deletion, removing exon 2 coding sequences (Rovinski *et al.* 1987). The result of the rearrangement is a truncated protein of 44kDa, which is lacking the N-terminal amino acid residues of p53. This construct immortalized cells with a lower efficiency than the p53 expressing constructs. Only intact p53-immortalized cells were transformed by secondary transfection with activated H-*ras*, therefore the 5'coding regions of the p53 gene are necessary for *ras* complementation but not immortalization. A genomic clone of p53, derived from a BALB/c mouse library and other p53 cDNA clones were capable of co-operating with *ras* to transform primary rat embryo fibroblasts (Eliyahu *et al.* 1984, Finlay *et al.* 1988). Due to its ability to co-operate with the *ras* oncogene in the transformation of primary cells, p53 was classed as a member of the nuclear oncogene family.

Evidence that wild-type p53 is a tumour suppressor gene rather than an immortalizing oncogene has been recently presented by several independent investigators. A p53 cDNA clone isolated from embryonic carcinoma cells failed to co-operate with the *ras* oncogene to produce transformed cells (Finlay *et al.* 1988, Eliyahu *et al.* 1988, Hind *et al.* 1989, Finlay *et al.* 1989). After comparison of several different p53 sequences it was possible to define a consensus wild-type (WT) p53 sequence (Finlay *et al.* 1988). It was found that p53 protein sequences which contained mutations, resulting from one or two nucleotide changes, that localise between 130 aa and 234 aa in the 390 aa protein, activated the p53 protein for co-operation with *ras*. The earlier studies, which implicated p53 as an oncogene, had analysed a mutant p53.

When WT p53 plasmids were co-transfected with combinations of oncogenes, eg. *myc*+*ras*, E1A+*ras*, E1A+E1B, mutant p53+*ras*, in primary rat embryo cells, there was a marked reduction in transformation, particularly when early passaged cells were transfected (Eliyahu *et al.* 1989). A Friend virus-induced erythroleukemic cell line, that showed complex rearrangement of p53 and did not produce p53 protein, was less tumourgenic than a normal p53-expressing erythroleukemic cell line, when injected into syngeneic mice (Hicks and Mowat 1988, Mowat *et al.* 1985). Mutations in the p53 gene, within the highly conserved region mentioned above, were detected in a significant proportion of human osteogenic sarcomas and colorectal carcinomas (Masuda *et al.* 1987, Baker *et al.* 1989). These results suggest that WT p53 expression has an adverse effect on transformed cells, and inactivation of p53 may be crucial in tumourigenesis.

p53 is believed to be essential in the control of cell proliferation. p53 was shown to be tightly regulated to the cell-cycle, when nondividing splenic lymphocytes only synthesized p53 4 hrs after stimulation by mitogen (Milner and Milner 1981). When nontransformed, quiescent 3T3 fibroblasts were restimulated to reenter the cell cycle by the addition of fresh serum, p53 mRNA and protein levels increased in

late G1, before the onset of DNA synthesis (Reich and Levine 1984). The induction of p53 protein synthesis was linked to cellular DNA replication in human peripheral blood lymphocytes, stimulated with phytohemagglutinin and interleukin 2, indicating that p53 was a cell-cycle dependent gene whose expression could be regulated by different mitogens (Mercer and Baserga 1985).

An indication of the importance of p53 during the cell cycle was presented, when it was shown that microinjection of monoclonal antibodies against p53 into the nucleus of quiescent Swiss 3T3 mouse cells, before stimulation with serum, inhibited the entry of the cells into S-phase and DNA synthesis. The p53 antibody had no effect if the microinjection was delayed until 4 hrs after stimulation, suggesting that p53 may be important in the G0 to G1 transition phase of the cell cycle (Mercer *et al.* 1982, Mercer *et al.* 1984). Transfection of plasmids encoding p53 antisense RNA inhibited growth of chemically transformed and untransformed mouse cells (Shohat *et al.* 1987). Microinjection of plasmids containing the p53 gene into quiescent Swiss 3T3, in the presence of incomplete serum, stimulated DNA synthesis (Kaczmarek *et al.* 1986b). These studies demonstrate that p53 can play a key role in controlling the proliferation of actively growing normal cells. The ability to affect DNA synthesis may be due to the intrinsic activity of non-sequence specific binding to double stranded and single stranded DNA (Steinmeyer and Deppart 1988).

As mentioned above, the SV40 large T antigen and the E1B-495aa protein both bind p53 to possibly inactivate its functions, predisposing the cell to transformation. This mode of action is similar to the inactivation of the retinoblastoma protein. The two functional domains of the SV40 large T antigen involved in transformation, bind the RB protein and p53 (DeCaprio *et al.* 1988, Schmieg and Simmons 1988). Similarly, both E1A, which binds the RB protein, and E1B-495aa, which binds p53, are required for transformation. Thus there

appears to be a correlation between RB and p53 binding and cellular transformation despite the separation of function within the E1 region of adenovirus.

1.8. *Transformation is the result of many complex virus-cell interactions*

Transformation of primary cells by adenovirus is caused by a culmination of many complex events, besides the probable inactivation of RB and p53 by E1A and E1B-495aa, respectively. Through the analysis of virus mutants, the expression of other regions of adenovirus have been found to influence the transformation ability of adenovirus. In summary then, some points will be reiterated. It has already been established above that E1A is central to transformation. However, viruses which have mutations in the E1B region within the genes encoding the 495aa or 175aa proteins, are defective for transformation (Jones and Shenk 1979b, Chinnadurai 1983, Bernards *et al.* 1986, Barker and Berk 1987, Herbst *et al.* 1988). Alterations in the E2A DBP enhanced transformation frequencies, due most likely to an activity possessed by the altered DBP, as viruses with total deletions of DBP transformed rat cells at frequencies similar to wild-type (Logan *et al.* 1981, Rice *et al.* 1987). Viruses containing mutations that map to E2B, particularly to the DNA polymerase, are defective for DNA replication in human cells and transformation of rat cells (Miller and Williams 1987). The main function of E3 is to protect the virus against host defence mechanisms during infection. E3 has been shown to be dispensable for the growth of virus in tissue culture, although the lack of E3 expression in viruses that do not express the E1A-13S product decreases the efficiency of focus formation by 4 (Kelly and Lewis 1973, Herbst *et al.* 1988).

One of the more important considerations of the induction of transformation is the host cell response to the invading virus, and how the intracellular environment is manipulated by the virus to favour uncontrolled growth. In permissive infections, when human adenoviruses infect established human cell lines, the lytic cycle is very efficient. The cells protein and DNA synthesis ceases, resulting in cell death

(Ginsberg *et al.* 1967). Replication of human adenoviruses is incomplete in cultured cells of non human origin. Replication in a semipermissive host, eg Ad2 infection of rat or hamster cells, is characterized by restricted viral replication and a 1000 fold reduction in virus yield (Gallimore 1974, Eggerding and Pierce 1986). Most cells will be productively infected with adenovirus and will lyse. Only a small number of cells will survive and become transformed. Nonpermissive adenovirus infection, eg Ad12 infection of hamster cells, is characterised by variable expression of early genes, no viral replication or late gene expression and no infectious progeny (Doefler and Lunholm 1970). Transformation (at a low frequency) only occurs in cells semi- or non- permissive for adenoviral growth. In these situations the virus has the opportunity to interact with cellular regulatory elements to induce cellular genes, possibly responsible for creating an intracellular environment leading to uncontrolled proliferation.

1.8.1. *Adenovirus regulation of cellular genes*

The interaction of adenovirus with the cell during infection results in alterations in cellular metabolism. Early in adenovirus 2 or 5 infection, before the onset of viral replication, the transcription of certain cellular genes is increased by the E1A products. Infection of HeLa cells increases the expression of the β-tubulin gene (Stein and Ziff 1984). The expression of heat shock protein (hsp) genes, particularly the 70kDa hsp gene (hsp70) are increased by 100-fold, but only 2-fold in the absence of E1A function (Kao and Nevins 1983). Hsp genes are a small number of specific genes induced in most organisms by brief heat treatment or other cellular stress (Schlesinger *et al.* 1982, Burdon 1986). The hsp70 gene is cell-cycle regulated. mRNA accumulates in the cytoplasm during the S of the cell cycle, and rapidly disappears during mid to late S and G2. This increase is reflected in the increase of protein in the nucleus (Kao *et al.* 1985, Wu and Morimoto 1985, Milarski and Morimoto 1986).

During infection, hsp70 is colocalised to the nucleus with E1A. A small fraction of hsp70 was found complexed with E1A (White *et al.* 1988). Perhaps the induction is in response to infection as a cellular stress. This is unlikely, as infection with an 9S virus (lacking the E1A-13S and 12S products), under conditions which result in a productive infection, did not induce hsp70 (White *et al.* 1988). Recently, hsp70 has been assigned the role of an ATP-dependent "unfoldase", responsible for folding incorrectly or incompletely folded proteins, by coupling ATP hydrolysis to disruption of quaternary structure until the protein assumes its correct configuration. Cytosolic hsp70 was also shown to maintain precursor proteins in a loosely folded state to translocate across membranes (Deshaies *et al.* 1988, Chirico *et al.* 1988). The significance of the induction of hsp70 by adenovirus infection is not well understood. Other members of the hsp gene family, hsp89 and hsp27, were not cell-cycle regulated or induced by E1A (Simon *et al.* 1987).

E1A products were also shown to activate the transcription of the β-globin gene and the rat preproinsulin gene when these genes were contained within a plasmid and transfected into E1A-expressing cells, or cotransfected with E1A-carrying plasmids cells (Green *et al.* 1983, Gaynor *et al.* 1984, Stein and Ziff 1984). The cellular β-globin and preproinsulin genes were not induced by E1A products, suggesting that conformation and accessibility of DNA may influence the induction ability of E1A products.

In E1-transformed cells, E1A reduced the expression of cellular proliferation-associated genes. Analysis of a number of Ad5 transformed rodent and human cell lines revealed that the expression of c-*myc* and another growth factor-inducible gene, the JE gene, was reduced at the level of mRNA accumulation (Timmers *et al.* 1988, Timmers *et al.* 1989). The JE gene product has recently been identified as part of a large family of secreted cytokine-like glycoproteins, secreted by monocytes and fibroblasts, after induction by a mitogenic or activation signal

(Rollins *et al.* 1988, Rollins *et al.* 1989). E1A-mediated transformation also suppressed the level of stromelysin mRNA (Offringa *et al.* 1988). Stromelysin is a secreted metalloprotease which degrades extracellular matrix components (Wilheim *et al.* 1987). The expression of stromelysin is induced by epidermal growth factor and transformation by polyoma virus, Rous Sarcoma virus and H-*ras* (Matrisian *et al.* 1985).

1.8.2 *Adenovirus induces cell-cycle associated genes*

In order to create an environment conducive for viral DNA replication, adenovirus induces quiescent cells to progress into S-phase, and growing cells to progress abnormally through the cell cycle, exhibiting a shortened G1 phase and DNA replication uncoupled from the synthesis of rRNA and polyamines (Braithwaite *et al.* 1981, Cheetham and Bellett 1982, Murray *et al.* 1982, Braithwaite *et al.* 1983). As a direct result of E1A expression, actin stress fibres disappear and some actin binding proteins are reorganized, resulting in a diffuse distribution of actin, resembling that found in early mitotic cells. The disruption of the cytoskeleton was found to be directly due to E1A and not to abnormal cell cycle progression induced by adenovirus (Jackson and Bellett 1985, 1989). As mentioned earlier, White and Cipriani (1989) suggest that specific disruption of intermediate filaments and the nuclear lamina is mediated by the 175 aa E1B protein.

To facilitate viral DNA synthesis, there is degradation of the cellular nucleoli (the site of synthesis for rRNA and assembly of ribosome subunits) and rRNA synthesis is inhibited during infection (Castiglia and Flint 1983, Lawler *et al.* 1989). The nucleolar components and small nuclear riboprotein-associated antigens are recompartmentalized to form pseudonucleoli, nucleated around sites of adenovirus DNA replication (Walton *et al.* 1989).

Concommitant with the induction of cell DNA synthesis, is the induction of cell-cycle associated genes by the E1A gene products. Adenovirus infection

preferentially activates cellular genes whose expression reaches a maximum in the late G1-S phase and are involved in DNA replication. Thymidine kinase activity and mRNA levels in rodent cells increased after Ad5 infection (Cheetham and Bellett 1982, Braithwaite *et al.* 1983). Ornithine decarboxylase activity was not increased after infection, although the mRNA level was increased (Liu *et al.* 1985). The transcription of thymidylate synthase, an essential enzyme in proliferating cells not provided with exogenous thymidine, was increased after infection (Navalgund *et al.* 1980, Zerler *et al.* 1987). Similarly, dihydrofolate reductase expression was induced (Yoder *et al.* 1983). The synthesis of proliferating cell nuclear antigen (PCNA, also known as cyclin), the auxiliary protein of DNA polymerase δ, was activated by E1A during infection (Prelich *et al.* 1987, Bravo *et al.* 1987, Zerler *et al.* 1987, Jelsma *et al.* 1989). The expression of histone H3, usually restricted to the S phase, was also activated during adenovirus infection (Hirschhorn *et al.* 1984b, Liu *et al.* 1985). Other enzymes associated with DNA replication, which are induced by adenovirus infection, include cellular topoisomerase 1, DNA polymerase, aspartate transcarbamylase, dCMP deaminase and ribonucleotide reductase (Chow and Pearson 1985, Ledinko 1968, Ledinko 1966, Zimmerman *et al.* 1970).

Late in infection there is a specific inhibition of translation of pre-existing mRNAs in the cytoplasm, and a decrease in the appearance of newly transcribed RNA in the cytoplasm, resulting in a shutoff of host cell protein synthesis, after viral DNA synthesis begins (Bello and Ginsberg 1967, Beltz and Flint 1979, Babich *et al.* 1983). The inhibition of translation may be due to the displacement of host mRNA from active polysomes (Philipson *et al.* 1975). The failure of cell mRNAs to be transported to the cytoplasm maybe mediated by a protein complex that includes the E1B-55K and E4-34K proteins (Babiss and Ginsberg 1984, Halbert *et al.* 1985). Virus infection does not block transport of all cellular mRNAs. The exceptions include the E1A-induced mRNAs of hsp70 and the β-tubulin mRNA family,

suggesting that induction by E1A overcomes the viral block, although translation of hsp70 was inhibited 20-fold (Moore *et al.* 1987).

Thus, adenovirus manipulation of host cell machinery is very complex. Most virus-host interaction is concerned with the deregulation of the cell cycle in order to establish an environment favourable for viral replication . Proteins encoded by the E1 region of adenovirus have been shown to bind to important cell-cycle regulation elements, eg. $p110^{RB}$, p300, p170 and p53, to disturb the cellular blocks of the cell-cycle, resulting in unrestrained growth, providing cellular constituents necessary for viral replication. Proteins from the E1A region transactivate the expression of cellular proteins involved in cellular DNA replication, in order to subvert the cellular machinery towards viral DNA replication. The role of some induced cellular genes, eg hsp70 and β-tubulin has not been well defined. In a small percentage of cells, viral replication is incomplete, the cells survive the encounter and become transformed, due to events that occured early in infection. The E1 region has integrated within the genomes of these transformed cells, and E1 products continue to maintain the transformed state by interacting with cell regulatory elements, through transactivation and repression of transcription.

1.8.3. *The strategies used to identify cellular proteins that are induced by or interact with the adenovirus early region*

Two general approaches have been used to increase the understanding of adenovirus interaction with the host cell. The first general approach has been to analyse the effect of E1A, by infection or transfection, on cellular genes known to be involved in functions which are modulated by adenovirus. An example is the analysis of DNA replication enzymes. Adenovirus infection was shown to induce cellular DNA synthesis in a variety of cells, and the induction of enzymes associated with DNA replication by adenovirus, was established soon after, as discussed in section 1.8.2. (Takahashi *et al.* 1966, Shimojo and Yamashita 1968). Similarly, the E1 region was known to transform primary cells, so the regulation of

cellular oncogenes, initially recognised as homologs of retroviral tumour-inducing genes, eg c-*myc*, c-*fos*, c-H-*ras*, after adenovirus interaction, was investigated. E1A products were responsible for the induction of c-*myc* and c-*fos*, depending on the type of cell assayed, but there was no induction of c-H-*ras*, although E1A could co-operate with an activated form of c-*ras* to fully transform primary cells. The response of genes encoding products induced in tumourgenesis were also analysed. For example, the activity of the gene coding for stromelysin, a secreted metalloproteinase associated with the progression of malignancy, was suppressed in E1-transformed cells (Matrisian *et al.* 1986, Ostrowski *et al.* 1988, Offringa *et al.* 1988).

1.8.3.i *Differential screening of cDNA libraries*

An alternative approach that could be used to identify cellular genes induced by E1A is to isolate cDNA representing cellular RNA species induced by E1A, by differential screening of a cDNA library of E1A-expressing cells. The technique involves the isolation of poly A^+ RNA from the cells of interest, synthesizing cDNA from the RNA, cloning the cDNA into expression vectors, resulting in a cDNA library, and screening duplicates of clones from the library with radiolabelled cDNA synthesised either from the original mRNA population, or an mRNA population from cells lacking the activity of interest. The cDNA clones that hybridise to both cDNA populations, represent mRNA that is common to both cellular populations. The rare cDNA clones that only hybridise to one cDNA population, represent mRNA that is unique to one cell population and warrant further investigation. The major problem with the isolation of selectively induced genes using this technique, is that it is often difficult to assign functions to the gene products, since they were not isolated by any functional assay. The technique of differential screening has been used in many laboratories with success to identify cellular genes induced during cell-cycle progression, tumourgenesis and viral induced transformation.

Differential screening of cDNA libraries has been used to identify cell-cycle dependent genes. Linzer and Nathans (1983) constructed a cDNA library of $1x10^6$ clones from poly A^+RNA present in BALB/c 3T3 cells after serum stimulation. After screening 3,500 clones differentially, 13 clones contained inserts which were expressed at higher levels in serum stimulated cells than in quiescent cells. One of the clones, designated proliferin, was characterised further and found to share sequence similarity with prolactin (Linzer and Nathans 1984). Proliferin was identified as part of the mitogen-regulated protein (MRP) family, although its functional role was not ascertained (Parfett *et al.* 1985).

Libraries were prepared from BALB/c 3T3 cells stimulated with platelet-derived growth factor (PDGF) for 4 hours (Cochran *et al.* 1983). From 8,000 clones differentially screened, 14 were shown to be G1 specific. The level of one clone, in particular, was dramatically increased by PDGF treatment and was called the JE gene. By sequence analysis and transient expression assays, the JE product was found to be a monocyte and fibroblast secretory cytokinelike protein (Rollins *et al.* 1989).

Cell-cycle dependent genes were also isolated from a differentially screened library made from G1 specific, temperature-sensitive hamster cells (Hirschhorn *et al.* 1984a). While the kinetics of induction has been studied for five clones, and three clones have been shown to be expressed at a higher level in leukaemias, the functions of the gene products are not known (Calabretta *et al.* 1985, Gibson *et al.* 1986). A similar situation exists with the 8 clones isolated from 8,000 clones from a library made from stimulated Swiss 3T3 fibroblasts (Edwards *et al.* 1985). One of the eight clones was similar in sequence to the murine B2 repetitive element, the level of which increases during transformation (Singh *et al.* 1985a, Grigoryan *et al.* 1985, Kohnoe *et al.* 1987).

Differential screening of cDNA libraries has also been used to identify genes involved in tumor progression. For example, Yamamoto and his colleagues (1983) isolated 31 clones out of 4,000 clones from a cDNA library made from azo-dye-induced rat ascites hepatomas. They demonstrated that a class of middle-repetitive sequences were specifically transcribed in a number of tumor cell lines. Steeg and her colleagues (1988a) isolated a gene (NM23) which was reduced in expression in metastatic melanoma cell lines, compared to the poorly metastatic melanoma cell line, by differential screening. The level of this gene was increased in rat embryo fibroblasts (REFs) cotransfected with Ad2 E1A and c-Ha-*ras*, which are nonmetastatic, compared to REFs transfected with c-Ha *ras*, which are highly metastatic (Steeg *et al.* 1988b).

A number of genes influenced by viral transformation have been isolated. For example, the SV40 large tumor antigen was shown to induce several cellular transcripts, isolated though differential screening of cDNA libraries made from SV40 transformed cells (Schutzbank *et al.* 1982, Singh *et al.* 1985b). Novel genes induced by Rous sarcoma virus (RSV) were isolated from a chick embryo fibroblast (CEF) cDNA library by differential screening using radioactive cDNA made from uninfected or RSV-infected CEFs and from a differentially screened RSV-transformed CEF library (Sugano *et al.* 1987, Bedard *et al.* 1987).

The differential hybridization technique was refined further to isolate cDNA representing differentially expressed mRNA in relatively low abundance. By removing cDNA representing mRNA common to both cell types, the cDNA library, and/or cDNA probes would be significantly enriched for the possibly rare cDNA representing the mRNA responsible for the activity of interest. This procedure is referred to as subtractive hybridization.

1.8.3.ii *Subtractive hybridization*

Scott and his colleagues (1983) hybridised radiolabelled cDNA made from a SV40 transformed BALB/c 3T3 cell line to cDNA from 3T3 cells immobilised to cellulose. The cDNA that did not hybridise was used to screen 6,400 clones, of which 42 clones were positive and were characterised into 4 major transcription units, shown to directly respond to the transforming protein of SV40.

A number of definitive experiments showed the value of subtractive hybridisation as a means to isolate low abundance mRNA. Davis and his group (1984b) hybridised cDNA from B cells against mRNA from T-cells and removed common sequences by passing the sample through hydroxyapatite, resulting in a cDNA probe highly enriched for B cell sequences. cDNA from T-cells was hybridised against mRNA from B-cell and similarly treated, resulting in a cDNA probe highly enriched for T-cell specific sequences. The probes were used to screen a cDNA library, highly enriched for T-cell specific sequences. 5,000 clones were screened, 35 clones were positive and they were able to identify the T-cell receptor from the positive clones by Northern and Southern analysis.

Subtractive cDNA libraries have been constructed in many experimental situations, when it is possible to obtain two closely related cell populations, which optimistically differ only in the activity of interest. Examples include the analysis of genes specifically expressed in growth-arrested cells compared to stimulated cells, and genes specifically expressed in non-metastatic as opposed to metastatic cell lines (Schneider *et al.* 1988, Dear *et al.* 1988). Subtractive cDNA probes have been used to screen cDNA libraries to isolate genes induced by transformation by oncogenes, eg transin (Matrisian *et al.* 1985), and cellular genes expressed at higher levels in adenovirus transformed cells (Kao and Nevins 1986).

As mentioned previously, the main problem with differential screening and subtractive hybridization, is the difficulty of assigning functions to the gene

products, since they were not isolated by functional assays. There are a number of steps involved in identifying the newly isolated gene. The cDNA is sequenced and the sequence compared to sequences held within databanks. Northern analysis to judge transcript size and induction kinetics is completed. From the result of the Northern, it may be necessary to screen a full length cDNA library for the full length cDNA, which can be used for *in vitro* translation to identify the protein product. Antibodies could be raised against peptide sequences. The cDNA or antisense cDNA could be microinjected, or transfected into the cell to investigate whether the phenotype of interest can be recreated or destroyed, respectively. This procedure does not necessarily reflect the correct function of the gene product, although a idea of the role of the protein may be gained. Thus as more sequences of genes induced in serum stimulation or transformation become identified and the function of gene products is deduced, it may be possible in the near future to understand the multitude of processes the cell undergoes, during progression through the cell-cycle or transformation.

1.9. *Scope of this Thesis*

Adenovirus transformation is the result of many complex interactions between the cell and the regulatory regions of adenovirus. The E1A region is essential for cellular immortalization, and together with the E1B region, can fully transform semipermissive cells. The manner in which the host cell responds to E1A products, to create an intracellular environment ultimately resulting in immortality, is not understood. Although E1A products can bind to cell-cycle regulatory elements, inducing the cell into S phase, and increasing the level of DNA synthesising machinery, the full scope of cellular responses to E1A products has not been elucidated. The aim of this thesis is to identify and characterise cellular genes induced by E1A products during the early stages of infection that may play crucial roles in the establishment of the immortalized phenotype. To achieve this goal, clones that were differentially screened from subtractive cDNA library, enriched for E1A-induced cellular sequences (constructed before the start of the current investigation), were characterized by sequence, Southern and Northern analysis in chapters 3 and 5. The transcription kinetics of the clones were also investigated in chapter 5. Chapter 4 deals with the identification and characterisation of a contaminating dependovirus, adeno-associated virus (AAV), found by sequencing induced clones from the subtractive library.

The effect of adenovirus infection on a cellular protein-degradation pathway, was also monitored. If a major protein degradation pathway was induced during adenovirus infection, unfamiliar proteins, for example virion components, may be recognised as foreign and rapidly degraded, leading to an inhibition of the lytic cycle, possibly aiding the establishment of a transformed state. To investigate this possibility the expression of ubiquitin, a major component of the ATP dependent degradation pathway for short-lived, damaged or denatured proteins, during adenovirus infection, was analysed in chapter 5.

A final discussion of the results and implications is presented in chapter 6.

Chapter 2

Materials and procedures

2.1. *Materials*

2.1.1 *Media and cells*

JC rats were bred in specific pathogen free conditions at the Animal Breeding Establishment, John Curtin School of Medical Research. HeLa cells were obtained from the American Type Culture Collection, Rockville. Human 293 cells were obtained from Dr. F. Graham, McMaster University, Ontario. Adenovirus-transformed cell lines, F17 and XHO-BRK, were obtained from Dr. P. H. Gallimore, University of Birmingham, England. All cell cultures were grown in Eagle's modified essential medium plus Earle's salts (Autopow, Flow laboratories Inc.). Foetal calf serum (FCS) was obtained from the Commonwealth Serum Laboratories, Aust, and heat inactivated at 56°C for 30min before use.

2.1.2 *Plasmids*

3'-Oligo (dG)-tailed pUC 9 was obtained from Pharmacia. pGem 1 was obtained from Promega Biotec. pSM620, containing the AAV genome cloned into the *Pst*1 site of pBR322, was kindly supplied by Dr. K. I. Berns, Cornell University, New York. pRBL26, containing the human ubiquitin coding region, was a gift from Dr. P.G. Board, JCSMR, Canberra. The plasmid containing a 2.1kb cDNA insert for human α-tubulin was originally obtained from Dr. N. J. Cowan, Princeton University, New Jersey. pE1A, containing 0-1571 nucleotides of WT; pE1B, containing 1831-3931 nucleotides of Ad 2 and pE3, containing 28962-32172 nucleotides of Ad2, were kindly provided by Dr. A. Braithwaite, JCSMR, Canberra. pE2, containing 21606-27372 nucleotides of WT was obtained from Dr. J. R. Nevins, Duke University Medical Center, North Carolina. pMR53, containing a 16-kilobase *EcoR*1 fragment of mouse genomic p53 was a gift from Dr. S. Benchimol, Ontario Cancer Institute,Ontario.

2.1.3. *Chemicals and DNA / RNA modifying enzymes*

SDS, TEMED and polyacrylamide (analytical grade) were purchased from Biorad. Genetic technology grade agarose was obtained from FMC. ^{32}P-labelled nucleotides were purchased from Amersham. All other chemicals used in experiments described in this thesis were of analytical grade, and obtained from the Sigma Chemical Company. Restriction enzymes, deoxy-nucleotide triphophates and Proteinase K were purchased from Boehringer Mannheim. DNase and RNase were obtained from Promega.

2.2. ***Procedures***

2.2.1 *Cell culture*

Establishment of primary rat embryo fibroblast cultures

Primary cell cultures of JC rat embryo fibroblasts (REF) were established by washing 15-17day old rat embryos extensively in phosphate buffered saline, (PBS, 0.137M NaCl, 6.75mM Na_2HPO_4, 2.5mM NaH_2PO_4), followed by mincing and trypsinization for 30 minutes in 0.125% (w/v) trypsin (dissolved in PBS) at room temperature (RT). The cell suspension was filtered through sterile gauze, centrifuged at 150g and resuspended in Autopow, supplemented with 10% (w/v) FCS. The cells were grown in 175cm^2 plastic tissue culture flasks (Falcon) at 37°C in Autopow+10%FCS, until a confluent monolayer of approximately 2x10^7 cells was achieved. Cells were passaged by washing throughly in PBS, exposing each flask to 2mls of 0.025% trypsin for 5min at 37°C, and inactivating the trypsin by adding 10mls Autopow+10%FCS. The cells in each flask were then subdivided into 5 more flasks, to produce a 1:5 dilution. The cells were passaged twice in this way, resulting in tertiary REFs (3°REFs).

Infection of 3° REFs

When confluent, the 3° REFs were infected with adenovirus. After removal of the medium, the cells were overlayed with 10mls Autopow-1%FCS containing virus estimated to be approximately 25 infectious units (iu) per cell, or without virus in the case of mock infection. The cells were incubated for 90mins at 37°C, after which time the original medium was replaced and the infected cells incubated at 37°C, until processed for RNA analysis, DNA analysis or transcriptional analysis.

Serum deprivation

To investigate the effects of adenovirus infection in the absence of serum, in some experiments media was changed to Autopow supplemented with 0.2% FCS when 3°REFs became confluent. The cells were serum deprived in this way for 24hrs before virus infection, and throughout virus infection.

2.2.2 *Viruses*

Virus mutants

The defects within adenovirus mutants are described in table 2.1. Adenovirus mutant strains dl 312, dl 313, dl 337, dl 338 were generous gifts from Dr. T. Shenk, Princeton University, New Jersey. Strains 12S PM961, E1A PM928, 12S virus, 13S virus and hr 3 were kindly supplied by Dr. E. Moran, Cold Spring Harbour, New York; dl 808 was obtained from Dr. G. Ketner, The John Hopkins University, Baltimore. The mutant dl 327 was from Dr. J.R. Cutt, Rutgers University, Piscataway, New Jersey.

Table 2.1. Defects of adenovirus mutants

Mutant	Mutation	Consequence	Reference
dl 312	Δ bp 448-1349 at 1.5 to 4.5mu	Δ 12S and 13S RNAs. no E1A proteins produced	Jones and Shenk,1979b
dl 309	Δ of 2bp at -22 and-23 from initiation site of VAI transcription at 29.5mu	WT for growth and E1A expression.	Jones and Shenk, 1979b Thimmappaya *et al.*, 1979
12S *	12S cDNA replaces E1A gene in dl 309	only 243 aa product	Moran *et al.*, 1986a
13S *	13S cDNA replaces E1A gene in dl 309	only 289 aa product	Moran *et al.*, 1986a
12Spm961*	point mutation at bp 961 guanine to adenine	glycine to lysine at 135aa in 243 aa product. Mutation in constant domain 2	Moran *et al.*, 1986b
E1Apm928*	point mutation at bp 928 thymine to guanine	cysteine to glycine at 124aa in both E1A products Mutation in constant region 2	Moran *et al.*, 1986b
hr 3	point mutation at bp1086 thymine to adenine	methionine to lysine at 186aa in 289aa product. Mutation in constant region 3	Harrison *et al.* , 1977 Glenn and Ricciardi, 1985
dl 313	Δ bp 1334 to 3640 at 3.5 to 10.5mu	No E1B proteins. E1A proteins fused to 3'exon of E1B	Jones and Shenk,1979b
dl 337 *	Δ bp 1770 to 1915	truncated E1B 21K protein	Pilder *et al.*, 1984
dl 338 *	Δ bp 2805 to 3329	truncated E1B 55K protein	Logan *et al.*, 1984 Pilder *et al.*, 1986
dl 327	Δ 78.5 to 84.3mu	Δ of Protein coding region E3, no E3 protein	Cutt *et al.*, 1987
dl 808	Δ of 2,008bp from 2946 to 932, numbered from right end of genome. 92-97.1 mu	Δ of open reading frames 2 to 7 of E4 in Ad 2 No E4 product, reduced levels of viral late RNA	Challberg and Ketner, 1981. Herisse *et al.*, 1981

* - constructed from dl 309

aa - amino acids

mu - map units

Δ - deletion

Virus propagation

Adenovirus type 5 (Ad5) and mutant strains dl 327 and dl 808 were grown and titrated on Hela cells. Human 293 cells, transformed by Ad5 and constitutively expressing adenovirus E1 proteins (Graham *et al.*, 1977), were used to propagate the E1 mutants dl 312, dl 313, dl 337, dl 338, hr 3, 12S PM961, 12S virus and 13S virus. The appropriate cell line was infected with virus at approximately 1 iu per cell. At 60hrs after infection the majority of cells had lost their adherent phenotype. Remaining adherent cells were dislodged by gentle agitation and the cellular suspension centrifuged at 1,200rpm (Beckman TJ-6) for 5min. The pellet of cells was resuspended in 20mM Tris-HCl at 1ml per original 175cm^2 flask. The cells were then frozen in dry ice for 5min before thawing at 37°C for 5 mins. This cycle was repeated twice before cellular debris was pelleted by centrifugation at

2,500rpm (Beckman,TJ-6) for 10 min. The supernatant, containing virus, was aliquotted and stored at -70°C.

Titrations of virus

Titrations of viruses were calculated by the fluorescent cell counting method of Philipson (1961). Cover slips within petri dishes were seeded with either Hela or 293 cells. When the cells were confluent, the media was removed and the coverslips were infected with a series of tenfold dilutions of virus in 10µl Autopow+1%FCS. After incubation at 37°C for 90min, media was replaced (Autopow+1%FSC) and the cells incubated at 37°C for a further 24 hrs. Each coverslip was washed twice in PBS, fixed in 3.7% formaldehyde in PBS for 10min, washed with PBS and water, consecutively, and fixed at -20°C in acetone for 5min . The coverslips were air dried and washed extensively with PBS, before reaction with V-antiserum (directed against adenovirus particles). V-antiserum was raised in rabbits according to Russell *et al.* (1967). The antiserum was diluted 1:10 in PBS, 100µl placed on each coverslip and incubated at 37°C for 30mins. After reaction with the primary antibody, the coverslips were extensively washed with PBS, before the secondary antibody was applied. 100µl of 1:20 dilution of fluorescein isothiocyanate (FITC)-conjugated sheep anti-rabbit antibody (Silenius Laboratory) was applied to the coverslips which were incubated at 37°C for 30mins. Coverslips were thoroughly washed with PBS and mounted using PBS : glycerol (50:50). To calculate the titre of virus per ml, approximately 10 fields of cells were viewed under UV and the fluorescent cells counted. The average number of cells was multiplied by the calibration factor of the Leitz microscope at the 40X magnification, divided by the dilution and the fraction of a ml used on the coverslip.

2.2.3 *Analysis of transcription rate by Nuclear Run on*

In order to investigate regulation of gene expression at the transcriptional level, nuclei were isolated from infected cells, nascent RNA chains were elongated by RNA polymerase, in the presence of a radiolabelled ribonucleotide and used to probe cDNA-containing plasmids immobilized on nylon membranes. The technique was adapted from Fahnestock and Lewis (1989) and Weinheimer and McKnight (1987). Approximately $5x10^6$-$2x10^7$ cells were collected by trypsinization and washed twice in PBS. Nuclei were isolated by lysis in 5mls 10mM Tris pH7.4, 10mMNaCl, 3mM $MgCl_2$, 3mM DTT, 0.5% Nonidet P40 (NP-40), 20units/ml RNAsin, for 3min on ice, followed by centrifugation at 1500rpm (Beckman TJ-6) for 10min. The nuclei were resuspended in 40µl storage buffer (10mM Tris pH7.5, 5mM $MgCl_2$, 2.5% Ficoll, 0.3mM spermidine, 1mM Dithiothreitol (DTT), 50% glycerol) and stored at -70°C. To prepare radiolabelled nuclear RNA, the

nuclei were incubated at 30°C for 30min with 200µCi α-^{32}P-dGTP, in a total volume of 100µl yielding a final concentration of 40mM Tris pH8.3, 150mM NH_4Cl, 0.5mM dATP, 0.25mM dUTP, dCTP, 7.5mM $MgCl_2$, 25% (v/v) glycerol. The DNA was digested by the addition of 12 units of RNAse-free DNAse and the reaction further incubated at 30°C for 10min. The nuclear matrix was degraded by the addition of 50µl 30mM Tris pH7.4, 6mM EDTA, 3%(w/v) SDS, 3mg/ml heparin, 1mg Proteinase K and incubation at 37°C for 1hr. The lysate was extracted with phenol:chloroform (1:1) and the organic phase reextracted with 100µl 50mM Tris pH7.4, 10mM EDTA, 0.5% SDS. To precipitate the RNA, 64µl of 10M ammonium acetate and 340µl isopropanol were included and the mixture incubated at -70°C for 15min. After centrifugation at 14,000rpm (Eppendorf) for 15min, the pellet of radiolabelled RNA was resuspended in 100µl of TE (10mM Tris-HCl pH7.5, 1mM EDTA) and denatured by the addition of 2µl 10M NaOH for 10min at 0°C. After neutralisation with 60µl 1M HEPES pH7.4, the RNA was precipitated with 720µl ethanol. The RNA was centrifuged at 14,000rpm (Eppendorf) at 4°C for 15min, resuspended in 500µl hybridisation mix, and added directly to the prehybridised filters.

Plasmid was bound to the filters by initially boiling 5µg of plasmid DNA per nuclear run-on in 0.25M NaOH for 5min in a volume of 50µl. In order to maintain denaturation of the plasmid, the samples were placed on ice for 5min, during which time 50µl of 0.25M NaOH, 0.125XSCC (1XSSC=150mM NaCl, 15mM sodium citrate) was added. The samples were then applied to a Zeta Probe membrane (Biorad), presoaked in water, immobilized in a 96 well Minifold II apparatus (Schleicher and Schuell), under vacuum. The apparatus enabled plasmid DNA to be immobilized in a slot configuration of 0.75mm x 8.0mm. Each well was rinsed with 300µl 0.25M NaOH, 0.125XSSC, under vacuum. Before prehybridisation the filters were rinsed in 2XSSC. Filters were prehybridised in 0.2mls/cm^2 of 50% formamide (freshly deionized), 1.5XSSPE (1XSSPE=180mM NaCl, 10mM sodium phosphate pH 7.7, 1mM EDTA), 1%SDS, 0.5% BLOTTO, 0.2 mg/ml yeast tRNA, 0.5mg/ml sheared salmon sperm DNA, at 50°C for 24hrs. Filters were hybridised with radiolabelled RNA for 48hrs at 50°C in 0.075ml/cm^2 of 50% formamide, 2XSSPE, 1% SDS, 0.5% BLOTTO, 10% dextran sulphate, 0.2mg/ml yeast tRNA, 0.2mg/ml sheared salmon sperm DNA and at least 5x10^6 cpm labelled RNA. Non-specific hybridisation was removed by initially washing filters in 2XSSC, 0.1% SDS for 10min at room temperature, followed by washing in 0.2XSSC, 1% SDS at 65°C for 10-30 mins. If non-specific radioactivity posed a problem after washing, the filters were rinsed in 2XSSC for 3x5 min, before exposure to 1µg/ml RNAse A in 2XSSC for 10mins at room temperature followed by 0.2XSSC, 1%SDS at 50° for 10min. The filters were sealed in polythene bags

and exposed to Kodak XAR-5 film with intensifying screens at -70°C for 7-30 days.

2.2.4 *Analysis of RNA*

Isolation of Total Cytoplasmic RNA

Confirmation of transcriptional regulation by E1A of specific genes was obtained by Northern analysis of poly A^+RNA, total cyoplasmic and nuclear RNA. Cytoplasmic RNA was prepared as described by Scott *et al.* (1983). All solutions sterilized by treatment with diethyl pyrocarbonate (dep), to inactivate RNAses, followed by autoclaving to decompose the dep (Palmiter,1974). Briefly, cells were collected by scraping and washed twice with PBS. The cells were resuspended in 0.1M NaCl, 10mM Tris HCl pH7.4, 1mM EDTA at a concentration of $1x10^7$ cells per ml at 4°C. $MgCl_2$ was added to a final concentration of 5mM, followed by the addition of NP40 detergent to 0.5%(v/v). The solution was centrifuged at 8,000rpm (Sorvall RC 5B) for 2 mins, DNA was isolated from the nuclear pellet as described in 2.6.i. and the supernatant extracted with an equal volume of water saturated phenol. The extraction was incubated at 65°C for 10min after the addition of EDTA to 25mM and SDS to 0.5% (w/v). In order to isolate the aqueous layer the extraction was centrifuged at 3,000rpm (Beckman TJ-6) for 10min. The cytoplasmic RNA fraction was then extracted with phenol:chloroform (1:1), chloroform and ether, consecutively. The RNA was precipitated by the addition of sodium acetate to 0.2M and 2.5 volumes of ethanol at -70°C for 16hrs. The RNA was pelleted by centrifugation at 10,000rpm (Sorvall RC 5B) at 4°C and resuspended in 1ml dep-H_2O. By measuring the absorbance of the RNA at 260nm and applying the conversion relationship (1 absorbance unit at 260 nm equals 40µg/ml RNA), it was possible to estimate the concentration of RNA. 20ug aliquots of total cytoplasmic RNA were re-ethanol precipitated and stored at -70°C for future Northern analysis.

Poly A^+ RNA preparation

Poly A^+ RNA was isolated from approximately 10-20mg of total cytoplasmic RNA essentially as described by Maniatis *et al.* (1982). Cytoplasmic RNA was ethanol precipitated, resuspended in 5ml binding buffer (0.5M NaCl, 10mM Tris HCl pH 7.4, 1mM EDTA, 0.1% SDS) and heated to 65°C for 5mins. After cooling to room temperature the solution was applied to a column containing oligo dT_{12-18} (Pharmacia), prewashed in 0.5M NaOH, sterile water and binding buffer. The eluate was reheated to 65°C, cooled and reapplied to the column. To eliminate any RNA nonspecifically bound to the column, 10 ml of binding buffer was applied to the column. All eluate fractions were saved for spectrophometric analysis. After the addition of 10ml elution buffer (10mM Tris HCl pH 7.4, 1mM EDTA, 0.1%

SDS) 1ml fractions were collected. The absorbances of all fractions were measured at 260nm and fractions containing poly A^+ RNA combined. The poly A^+ RNA fraction was ethanol precipitated and stored at -70°C.

Isolation of Nuclear and Cytoplasmic RNA

Nuclear and cytoplasmic RNA were isolated according to the procedure of Wilkinson (1988). Collected cells were washed twice in ice cold Tris-saline (25mM Tris pH 7.4, 130mM NaCl, 5mM KCl, and resuspended in 4 mls ice-cold Tris-saline. In order to lyse the cellular membrane, 1ml of ice-cold 1% NP-40, 0.5% sodium deoxycholate, 0.02% dextran sulphate was added and the tube gently inverted. After centrifugation at 2500g for 5min at 4°C, the supernatant, (the cytoplasmic fraction), was transferred to a tube containing 5mls of phenol:chloroform (1:1). The SDS concentration was increased to 1% and 150 µl of 5M NaCl was introduced. The extraction was vortexed thoroughly and centrifuged at 2500g for 10 min at room temperature. The phenol:chloroform extraction was repeated three times, followed by a chloroform extraction and an ether extraction. Finally the cytoplasmic RNA was precipitated with 10mls of absolute ethanol at -70°C.

The pellet of nuclei was resuspended in 1ml of guanidinium lysis buffer (4M guanidinium isothiocyanate, 1M ß-mercaptoethanol, 25mM sodium acetate pH 5.2) and vortexed for 15 sec to lyse the nuclei. The lysate was layered over 5.7M cesium chloride/2mM EDTA in an ultracentrifuge tube at a volume ratio of 1.5:1 (lysate:CsCl). The gradient was centrifuged at 150,000g for 16 hrs at 20°C. The RNA pellet was resuspended in 400µl water and extracted twice with an equal volume of chloroform:1-butanol (4:1). To precipitate the nuclear RNA, 50µl of 3M sodium acetate pH 5.2 and 1ml of absolute ethanol was added, and the sample stored at -70°C.

Northern analysis

Northern analysis was performed as described by Ceredig *et al.* (1989). Aliquots of 20 µg total cytoplasmic RNA, or 5µg poly A^+ RNA were ethanol precipitated and resuspended in 3.7µl water. 3µl of RNA Ladder (Pharmacia) was also included to be processed. Deionised glyoxal was added to a final concentration of 1M. The final buffer also contained 50% (v/v) deionised dimethyl sulfoxide (DMSO) and 10mM sodium phosphate pH 6.7 in a total volume of 16µl (Maniatis et al., 1982). The mixture was incubated at 50°C for 60min, before the addition of 4µl loading buffer (50% glycerol, 0.01M sodium phosphate pH 6.7, 0.4% bromophenol blue). The samples were electrophoresed in 1% agarose 0.01% sodium phosphate at 3-4V/cm. The electrophoresis buffer was circulated to maintain a constant pH. The RNA within the gels was stained by 30µg/ml acridine

orange in 10mM sodium phosphate for 10min followed by destaining in 10mM sodium phosphate at 4°C overnight and photographed under UV light using Polaroid 57 film, to ensure equal loading of RNA (McMaster and Carmichael 1977). Alternatively the RNA was transferred from the gel, without prior treatment, to a prewet nylon membrane (Zeta Probe) in 10mM NaOH, as described by Vrati *et al.* (1987). After transfer for 16hrs, the membrane was rinsed in 2XSSC and air dried before prehybridisation.

Northern prehybridization and hybridization.

Prehybridization and hybridisation to Zeta-Probe membranes were essentially performed as described in the manufacturers instruction manual. Briefly, the membranes were prehybridized at 50°C for 4hrs in 2ml/10cm^2 50% (v/v) deionized formamide, 2XSSPE, 7% (w/v) SDS, 0.5% (w/v) BLOTTO, 0.25mg/ml yeast tRNA and 0.25 mg/ml sheared salmon sperm DNA. Membranes were hybridised at 50°C for 16 hrs in 1ml/10cm^2 50% (v/v) deionized formamide, 2XSSPE, 1% (w/v) SDS, 0.5% (w/v) BLOTTO, 0.25mg/ml yeast tRNA, 0.25 mg/ml sheared salmon sperm DNA, 10ng/ml ^{32}P-labelled DNA probe (specific activity 2x10^8-1x10^{9}cpm/µg) and 10% (w/v) dextran sulfate (Wahl *et al.*, 1979). In order to remove nonspecific hybridization, the membranes were washed with vigorous agitation in 2XSSC ,0.1% SDS for 20 min RT, followed by 0.5XSSC, 1% SDS at 65°C for 15-60min, until radioactivity detected by a hand held geiger counter was between 10 and 2 cps. The membranes were sealed within plastic bags and exposed to Kodak XAR-5 film with intensifying screens at -70°C for 1-7 days. To reprobe the membranes, the membranes were stripped of probe by immersion in 0.1XSSC, 1%SDS at 100°C and agitation for 1 hr at RT. To quantitate RNA levels the membranes were reprobed with α-tubulin, the expression of which is unaffected by the expression of the E1A region of adenovirus (Roberts *et al.* 1985). To compare RNA levels the autoradigraphs were scanned using a LKB UltraScan XL densitometer.

2.2.5 *Rescreening the subtracted library*

Synthesis of cDNA from mRNA as a probe

It was necessary to rescreen cDNA clones isolated from a subtractive library (as described in Appendix 1) to confirm that the clones were induced by adenovirus infection. Radiolabelled cDNA was synthesised using poly A$^+$ RNA isolated from cells uninfected, or infected with adenovirus WT or dl 312 as a template, to probe plasmid DNA immobilised on nylon membranes. 2.5µg of poly A$^+$ RNA was ethanol precipitated and resuspended in 12µl water, to which was added 2.5µl oligo (dT), 0.2mg/ml . The template RNA was denatured by heating the mixture at 80°C for 90secs, before chilling in ice-water. To the denatured template/primer mix

was added 3µl reaction buffer (50mM Tris-HCl pH8.3, 20mM DTT, 7.5mM MgAcetate, 0.1mM each of dATP, dGTP, dTTP and 0.02mM dCTP), 1µg BSA and 20µCi α-^{32}P dCTP. The reaction was mixed before, and after the addition of 3units of AMV reverse transcriptase. The reaction was incubated at 42°C for 60min and quenched by the addition of EDTA to 0.02M and Sarcosyl to 0.4%. A small aliquot was subject to thin layer chromotography to judge success of the reaction. The cDNA (specific activity 2-5x10^5cpm/µg original RNA) was precipitated by the addition of ammonium acetate to 2M and 2 vols of ethanol, followed by freezing in liquid nitrogen and centrifugation at 14,000rpm (Eppendorf) for 15min at 4°C. The pellet was resuspended in the carrier DNA solution used for hybridisation of plasmid-bound membranes.

Preparation of plasmid bound membranes for rescreening

Plasmid-immobilised membranes were prepared by infecting each well of a 96 well plate (Linco) with a monoclonal colony of bacteria containing the cDNA insert and plasmid in the presence of ampicillin (amp). The colonies were left at 37°C for 16hrs and were subsequently transferred, using a "bed of nails", to Hybond nylon membranes which were prelayered on amp-plates. The colonies were allowed to regenerate on the membrane at 37°C for approximately 6hrs, before processing of the membranes to expose plasmid DNA for screening. The membranes were laid on Whatman 3MM paper saturated with 0.5M NaOH, 1.5M NaCl for 5min, Whatman 3MM saturated with 0.5M Tris pH 7.5, 1.5M NaCl for 5 min, and Whatman 3MM saturated with 2XSSPE for 5 min, consecutively, before fixation by baking in vacuo for 90min at 80°C. Membranes were prehybridised at 68°C for 4hrs in 5XSSPE, 0.2% SDS, 0.2% BLOTTO, 0.2mg/ml sheared salmon sperm DNA. The hybridisation mix was essentially the same, with the addition of radiolabelled cDNA. Membranes were hybridised for 16hrs at 68°C. Removal of non-specific hybridisation was achieved by washing the membranes in 2XSSC, 0.1% SDS for 15 min followed by 15 min washes in 0.5XSSC, 0.1% SDS and 0.1XSSC, 0.1% SDS at RT. The membranes were sealed within plastic bags and exposed to Kodak XAR-5 film with intensifying screens at -70°C for 1-2 days.

2.2.6. *Genomic DNA analysis*

Analysis of specific DNA sequences directly from whole cells

A modification of the method described by McIntyre and Stark (1988) was used to assay for specific DNA sequences from whole cell lysates. 1x10^5 cells were infected with 25iu of virus per cell for 24hrs. Cells were harvested by trypsinization and washed with PBS. The cell pellet was resuspended in 250µl of 0.4M NaOH and heated at 80°C for 10min. Cellular debris was pelleted by centrifugation at 14,000rpm (Eppendorf) for 5min and 100µl of the supernatant

was applied to a Zeta Probe membrane. The membrane was prewetted in 0.4M NaOH and immobilised in a slot blot manifold (Schleicher & Schuell), before application of the samples. After the solutions passed through the membrane, each well was rinsed with 100µl 0.4M NaOH. Upon completion, the membrane was rinsed twice with 2XSSC for 5 min and allowed to dry for at least one hour at RT, before prehybridisation and hybridisation as described in 2.6.iv.

Isolation of Genomic DNA

To examine gene size and obtain preliminary information about gene structure, the isolation of genomic DNA of high molecular weight, relatively free of protein contaminants was essential for Southern analysis. Genomic DNA was isolated from nuclear pellets of cells subjected to cytoplasmic RNA extraction (section 2.4) using standard techniques. Basically, the nuclear pellet was resuspended in 4.5mls of a hypotonic solution (10mM Tris pH7.4, 10mM NaCl, 10mM EDTA), to which was added SDS to 1% and 2mg of proteinase K. This mixture was incubated at 37°C for 16hrs. Sodium perchlorate was added to a final concentration of 1M in a total volume of 6mls and incubated at 37°C for 30 min with gentle agitation. The mixture was extracted with phenol:chloroform (1:1) twice, followed by a chloroform extraction.At this stage, the DNA was precipitated by the addition of $^1/_{10}$ volume of 3M sodium acetate and 2.5 vols. ethanol. DNA was collected by "spooling", washed with 70% ethanol and resuspended in 1ml water/$2x10^7$cells, resulting in an approximate concentration of 1mg/ml. The concentration of DNA was estimated by measuring absorbance of a diluted sample at 260nm.

Isolation of viral DNA

To isolate viral DNA, crude cell lysates, containing virus particles were digested with micrococcal nuclease (S7, Boeringer Mannheim), which degrades cellular DNA and is unable to digest encapsidated viral DNA. After inactivation of the nuclease, lysates were incubated with proteinase K (Boeringer Mannheim), to remove the viral coat proteins, phenol extracted, and the viral DNA ethanol precipitated. 0.9mls of virus containing lysate was digested with 150 units Micrococcal nuclease (reconstituted at 15units/µl in 50mM Tris pH7.9, 10mM NaCl, 1mM EDTA, 0.5mM DTT, 0.1% NP-40, 50% glycerol) in 60mM KCl, 15mM NaCl, 20mM Tris pH7.8 and 2mM $CaCl_2$ at 25°C for 2hrs. The enzyme was inactivated by the addition of 50µl 0.5M EGTA, before the addition of 1mg of proteinase K and incubation at 37°C for 15min. The reaction was extracted with an equal volume of Tris pH8-saturated phenol, followed by chloroform extraction. DNA was precipitated by the addition of 1/10 vol. 3M sodium acetate and 2.5 vol. ethanol, with centrifugation at 14,000rpm (Eppendorf) for 30 min. The DNA pellet was resuspended in 100µl water and stored at 4°C.

Southern Analysis

Southern transfer was initially described by Southern (1975), as a procedure to identify specific sequences among DNA fragments, generated by restriction enzyme digestion and separated according to molecular weight by submerged horizontal gel electrophoresis. To establish the cDNA clones as cellular, 15µg aliquots of genomic DNA were digested with 100units of *EcoR*1 and *Hind*III in 1XKGB buffer (100mM potassium glutamate, 25mM Tris-acetate pH7.6, 10mM magnesium acetate, 50µg/ml BSA, 0.5mM 2-mercaptoethanol, McClelland *et al.* 1988) at 37°C for 16hrs. The digestion was arrested by the addition of $^1/_5$ digestion volume of 5X layering solution (50mM Tris,pH8.0, 100mM EDTA, 1% sarcosyl, 7.5% ficoll 400, 0.05% bromophenol blue, 0.05% xylene cyanol) and electrophoresed through 0.8% agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA) containing 0.5µg/ml ethidium bromide at 7 volts/cm. After 3-4hrs, when the bromophenol blue was approximately 2cm from the end of the gel closest to the anode, electrophoresis was terminated, the gel was photographed and prepared for DNA transfer to a nylon membrane as described by Reed and Mann (1985). Briefly, the gel was gently rocked in 0.25M HCl for 2x5min, until the bromophenol blue became tinged slightly with yellow. The gel was then carefully laid on a 4cm wick of Whatman 3MM paper, presoaked in 0.4M NaOH, within a plastic 12x20cm lunch box. Zeta probe membrane, presoaked in distilled water, was layered on the gel. After the elimination of visible bubbles, Whatman 3MM paper was placed on top, and paper towels stacked to the level top of the plastic box. The lid was snapped on, providing slight pressure on the paper towels. Transfer proceeded for 5-16 hrs. After transfer the membrane was soaked in 2XSCC and air dried.

Southern and slot blot prehybridisation and hybridisation

Probing DNA-containing Zeta Probe membranes with radioactive labelled DNA was essentially as described in the manufacturer's manual. Membranes were prehybridised for 4 hrs at 65°C in 0.5mls/cm^2 membrane of 2XSSPE, 7% SDS, 0.5% BLOTTO, 0.5mg/ml sheared salmon sperm DNA. Membranes were hybridised at 65°C for 16hrs in 0.1ml/cm^2 membrane of 2XSSPE, 3% SDS, 0.5% BLOTTO,10% dextran sulphate, 0.5mg/ml sheared salmon sperm DNA, 10ng/ml α-^{32}P labelled DNA probe (specific activity 2x10^8-1x10^{9}cpm/µg). After hybridisation, the membranes were washed initially in 2XSCC, 0.1% SDS at RT for 10 min, followed by 0.5XSSC, 1% SDS at 65°C for 30min. If non-specific hybridisation still posed a problem, the filters were washed in 0.1XSSC, 1%SDS at 65°C for 30 min.The membranes were sealed within plastic bags and exposed to Kodak XAR-5 film with intensifying screens at -70°C for 1-7 days.

2.2.7 *Construction of radiolabeled DNA probes*

Oligolabelling

In order to generate radioactive DNA probes for hybridisation, DNA inserts were internally labelled with ^{32}P by oligonucleotide priming, as described by Hodgson and Fisk (1987). 100ng of template DNA was combined with 60ng P28 universal primers (supplied by the Medical Molecular Biology Unit, John Curtin School of Medical Research) in a total volume of 4µl. The mixture was sealed in a siliconized glass capillary and placed in a boiling water bath for 2min, followed by immersion in ice. The mixture was released into an eppendorf containing 7µl water, to which was added 2µl of 10X oligolabelling buffer (0.5M Tris pH6.9, 0.1M magnesium sulfate, 1mM dithiothreitol, 0.6mM each of dATP, dGTP, dTTP), 140µCi $\alpha^{32}P$-dCTP and 10 units of Klenow fragment of DNA polymerase (Bresatec Aust.) to a volume of 20µl. After incubation at 25°C for 60min, the reaction was terminated by the addition of 80µl 0.1%SDS in TE. 1% of the reaction was subject to thin layer chromatography. 100µg of sheared salmon sperm DNA was added to the remaining fraction before ethanol preciptation by the addition of 1/10 vol of 10M ammonium acetate and 2.5vols ethanol. After centrifugation for 15min at 4°C, the precipitate was resuspended in 10µl water, and boiled in the presence of 2.5mg sheared salmon sperm DNA. Immediately after boiling the probe was added to the hybridization mixture and added to the prehybridised, bagged membrane.

Nick translation

Previous to the use of random priming as a means of radiolabelling DNA, DNA was radiolabelled by nick translation by a modification of the technique described by Rigby *et al.* (1977). A typical reaction mix contained 200ng DNA, 50mM Tris-HCl pH7.5, 7.7mM magnesium acetate, 4mM DTT, 3µg BSA and 2.5ng DNase I in a volume of 30µl. This mix was incubated at 14°C for 15min. To repair nicks introduced in the initial incubation, 0.0025mM each dATP, dTTP and dGTP, 20µCi α-^{32}P dCTP and 5 units DNA polymerase 1 (Klenow fragment, Bresatec Aust) were added in a final volume of 41µl and incubated at 14°C for 15min. The reaction was terminated by the addition of 12mM EDTA and 0.5% SDS. 0.1% of the product was subject to thin layer chromatography. To recover radiolabelled DNA, 10µg of sheared salmon sperm DNA was added and the DNA precipitated by the addition of 10µl of 10M ammonium acetate and 2.5vol ethanol. After freezing in liquid nitrogen, the DNA was pelleted by centrifugation at 14,000rpm (Eppendorf) at 4°C for 15min. The pellet was resuspended in 20µl water, the appropriate amount of sheared salmon sperm was added and boiled for 5min before addition to hybridisation mix.

Thin layer chromatography

Thin layer chromatography was utilised to assay for incorporation of radiolabel in DNA. An aliquot of radiolabelled DNA was spotted on one end of a strip of PEI-cellulose (Merck). The strip was placed in a beaker containing a small amount of chromatography solvent (0.75M potassium phosphate, pH3.5), ensuring that the spot of DNA was above the miniscus of the solvent. The beaker was sealed with plastic film, and the chromatogram allowed to develop until the solvent front was 1cm from the top of the strip. The strip was dried, wrapped in plastic wrap and exposed to x-ray film for 30min. Incorporated label remained at the origin, while unincorporated label migrated closely behind the solvent front. By scraping PEI from areas of radioactivity into scintillation vials for Cherenkov or liquid scintillation counting, the level of incorporation and specific activity was quantitated.

2.2.8. *Plasmid and cDNA insert preparation*

Transformation of plasmid into bacteria

To obtain DNA from the cDNA insert in sufficient amounts to be used as probes and in nuclear run on experiments, it was necessary to amplify the cDNA containing plasmid and isolate the insert through low melting point agarose. Initially, the plasmid of interest was transformed into competent *E.coli* strain MC1061.1 (amp^S tet^R). Amplification of plasmid DNA was essentially as described by Birnboim and Doly (1979), with modifications as suggested by Ish-Horowicz and Burke (1981).

Bacterial cells were made competent by a modification of the procedure described by Maniatis *et al.* (1982). Basically, 500mls of Luria broth (LB) was inoculated with a single colony of MC1061.1. Growth, at 37°C with shaking, was monitored until the absorbance of the culture reached 0.6 to 0.8 at 600nm, indicative of maximum density of cells while still in a logarithmic phase of growth. The culture was chilled at 0°C for 10min and centrifuged at 8,000g for 10min at 4°C. After removal of the supernatant, the cells were resuspended in 50mls ice-cold 0.1M $CaCl_2$ and incubated at 0°C for 20min. Cells were harvested by centrifugation at 8,000g for 5min, resuspended in 5mls ice-cold 0.1M $CaCl_2$:glycerol (85:15) and aliquoted into prechilled eppendorf tubes (100µl/vial) before freezing in liquid nitrogen and storage at -70°C. Transformation by plasmid was accomplished by the following technique. An aliquot of competent cells was thawed in ice water for 10mins before the addition of 1.8mls ice-cold 0.1M $CaCl_2$. 100µl aliquots were transfered to chilled eppendorfs and 1µl of plasmid DNA (ranging in concentration from 0.5ng/µl to 100ng/µl), or 5µl ligation mix, was added. After mixing gently, the cells were incubated at 0°C for 30min, heat shocked at 43°C for 45secs and returned to ice-water, before the addition of 1.4mls of LB and incubation at 37°C

for 1hr. The cells were pelleted by centrifugation at 1,500g for 2 mins and resuspended in 300µl LB. Dilutions were spread onto LB agar plates containing 40µg Amp/ml LB (amp-plates). All plates were incubated overnight at 37°C. Competent cells made by this procedure produced between $5x10^6$ to $1x10^8$ transformants per µg plasmid DNA.

Plasmid DNA preparation

Before amplification of the plasmid it was necessary to determine whether newly transformed colonies contained the insert-containing plasmid. Small amounts of the plasmid were made by initially growing a single colony in 5mls of LB+Amp, and exposing the cells to alkaline lysis, a scaled down version of the technique described below (phenol extraction and ethanol precipitation instead of cesium chloride gradients). After confirming that the plasmid contained the appropriate insert, by digestion with appropriate restriction enzymes, large amounts of plasmid were produced through alkaline lysis (Birnboim and Doly 1979, Ish-Horowicz and Burke, 1981).

A single colony of bacteria was initially inoculated into 10 mls LB+40µg/ml amp, and grown at 37°C for 8hrs. This slurry of bacteria was inoculated into 500mls LB+40µg/ml amp and incubated at 37°C overnight, with agitation. The cells were collected by centrifugation at 5,500rpm for 8min (GSA rotor,Sorvall RC5B), resuspended in 5ml/250ml LB lysozyme solution (50mM glucose, 25mM Tris pH 8.0, 10mM EDTA and 8mg/ml lysozyme).and incubated at RT for 5min. The lysed bacteria were transferred to a Beckman SW27 polyallomer tube in 5ml aliquots, to which 20mls 0.2M NaOH, 1% SDS was added, inverted and incubated at 0°C for 10min. To neutralise the mixture, 15mls of ice-cold 5M potassium acetate pH4.8 was added and placed on ice for 10min. Cellular debris was pelleted by centrifugation at 20,000rpm for 20min at 4°C in a Beckman SW27 rotor. DNA within the supernatant was precipitated by the addition of 0.6vol isopropanol, incubation at RT for 15min and centrifugation at 10,000rpm for 30min in 35ml corex tubes in a Beckman SW27 rotor. The pellet was washed in 70% ethanol, before resuspension in 8mls TE pH 8.0. To achieve a density of 1.55gm/ml, 8gms of cesium chloride was dissolved in the DNA mixture. Ethidium bromide was added to a final concentration of 0.75mg/ml, and the plasmid preparation was transferred to ($^5/_8$" X3") quick seal Beckman tubes, sealed and centrifuged at 40,000rpm at 20°C for 60hrs in a 50Ti rotor. After centrifugation, the plasmid banded midway between the protein debris at the top of the tube, and the RNA pellet at the bottom of the tube. The band was collected and extracted with TE-saturated isopropanol until all ethidium bromide was removed from the band. The plasmid solution was dialysed against TE pH8.0 for 16hrs. After dialysis the

concentration of plasmid was calculated from measuring optical density at 260nm. and stored at 4°C.

Isolation of DNA from low melting point agarose

cDNA inserts were isolated from plasmids before use as probes, by digestion with an appropriate restriction enzyme, separation by electrophoresis through agarose, and purification from low melting point agarose. Approximately 150µg plasmid DNA was digested for 16 hrs with 100units of specific enzyme in 1XKGB to cleave out the insert from the plasmid. The digestion was terminated by the addition of 1/5 vol 5X stop buffer before heating at 65°C for 5min. The mix was then loaded on a 1% low melting point horizontal agarose gel and electrophoresed at 4 volts/cm in TAE until the bromophenol blue dye migrated two thirds the length of the gel. The gel was then stained with 0.5µg/ml ethidium bromide and the fragment of interest excised under UV. The gel slice was equilibrated with 1ml of NAS (0.25M NaCl, 0.24M sodium acetate and 0.5% SDS) for 5min. NAS was removed and the gel melted at 65°C for 5 min. An equal volume of NAS was added and allowed to stand at 65°C for 15 mins. The molten gel was extracted with freshly prepared phenol (saturated with 1M Tris-HCl pH8.0), at 65°C for 2min before centrifugation at 8,000g for 5min at 4°C. DNA was precipitated from the aqueous phase by the addition of $^1/_{20}$ vol of 3M sodium acetate and 2.5 vol ethanol, incubation at -20°C for 30min and centrifugation at 8,000g for 30min at 4°C. The precipitate was resuspended in 50µl water before another ethanol precipitation to reduce the high sodium salt concentration. Ammonium acetate was added to 3.75M and 2.5 vol ethanol was added. The sample was centrifuged for 30min at 8,000g at 4°C. The pellet was resuspended in 50µl water, and DNA concentration estimated by electrophoresis through agarose with a DNA standard of known concentration.

2.2.9.*Sequencing cDNA inserts*

Subcloning cDNA inserts into pGEM1

To identify cDNA inserts as previously described or unknown genes, it was necessary to sequence the clones. To exploit an established sequencing regime, the cDNA inserts were subcloned into pGEM1 (Promega Biotec.). Initially, 1µg of plasmid was linearised by a restriction enzyme and dephosphorylated to minimise self ligation. The plasmid was digested with a 20 fold excess of *Pst* 1 restriction enzyme for 2 hrs at 37°C, in the presence of a medium salt buffer (50mM NaCl, 10mM Tris-HCl pH7.5, 10mM $MgCl_2$, 1mM DTT, 0.1mg/ml BSA) in a final volume of 21µl. The plasmid was dephosphorylated by the addition of 1unit alkaline phosphatase (calf intestine) with 0.5µl 20% SDS and 3.5µl 1M Tris-HCl pH 9.0, followed by consecutive incubation at 37°C for 30min and 50°C for 30min.

To terminate the reaction, EDTA was added to a final concentration of 16mM. The sample was extracted with an equal volume of phenol equilibrated with 10XTAE and chloroform. DNA was precipitated by the addition of 7µl 10M ammonium acetate and 70µl ethanol, freezing in liquid nitrogen and centrifugation at 14,000rpm (Eppendorf) for 15min at 4°C. The pellet was redissolved in 20µl water, yielding approximately 50µg/ml linearised dephosphorylated plasmid.

cDNA inserts were successfully ligated into pGEM when 13ng of insert was ligated to 150ng linearised plasmid (a molar ratio of 1:3), using 1unit T_4 ligase in the presence of 50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 0.5mglml BSA, 5mM DTT, 1mM spermidine, 1mM ATP for 16 hrs at 17°C. Transformation proceeded and plasmids of interest were isolated, verified and amplified as described in 2.2.8.

Sequencing pGEM plasmids

The pGEM vector contains both the T7 and SP6 RNA polymerase promoters, flanking a multiple cloning site, containing 11 unique restriction enzyme sites. Plasmids were sequenced according to the manufacturers instructions, using the GemSeq K/RT system (Promega Biotec.). Basically, 2µg plasmid DNA, in a total volume of 22µl, was alkali denatured by incubation for 5min at RT in 0.2M NaOH, 0.2mM EDTA. The solution was neutralized by the addition of 3µl sodium acetate pH5.2, the volume increased by the addition of 7µl water, and the DNA precipitated by the addition of 75µl ethanol, chilling at -70°C for 5min and centrifugation at 10,000g for 5min. The pellet was resuspended in 6µl water, to which was added 1µl of 10Xbuffer (100mM Tris-HCl pH7.5, 500mM NaCl) and 3µl of 10ng/µl of either the T7 promoter primer or the SP6 promoter primer. The plasmid and primer were annealed at 37°C for 60mins. In preparation for the sequencing reactions, 3µl of the appropriate nucleotide mix, supplemented with a limited amount of a different dideoxy NTP, was placed into four tubes labelled A,T,C,G for each set of reactions. 5units of Klenow DNA polymerase were included in the annealing reaction, and mixed gently with the addition of 4µl α-^{32}P dATP (10mCi/ml). 2.8µl of annealing mix was added to each nucleotide solution, mixed gently and incubated at 37°C for 15mins. The nascent DNA chains were further extended by the addition of 1µl chase solution (0.125mM each of dATP, dCTP, dGTP, and dTTP), and incubation at 37°C for 15min, ensuring that the chains where no dideoxynucleotide was incorporated were polymerised to a high molecular weight.. The reactions were terminated by the addition of 5µl stop buffer (90% formamide, 20mM EDTA, 0.3% bromophenol blue, 0.3% xylene cyanol) and electrophoresed as described below.

pUC sequencing

A number of plasmid inserts were sequenced, without subcloning into pGEM by the method described in the manual in the pUC Sequencing Kit (Boehringer mannheim) 2µg plasmid DNA was denatured in 40µl 0.2M NaOH, 0.2mM EDTA as described above. 4µl 2M ammonium acetate pH4.5 was added and the plasmid precipitated by the addition of 100µl chilled ethanol and centrifugation at 10,000g for 20min at 4°C. To the dried DNA pellet was added 2.5pmol M13/pUC sequencing primer or M13/pUC reverse sequencing primer, 1.5µl 10X Reaction Buffer (100mM Tris-HCl pH7.5, 500mM NaCl) and 10µCi α-^{32}P dATP to a total volume of 15µl. The annealing mixture was incubated at 37°C for 15min. 2µl of each corresponding dNTP/dideoxyNTP mixture was pipetted into four Eppendorf tubes labelled A,T,G and C. To the annealing reaction was added 2 units of Klenow enzyme. After mixing by repeated suction with the pipette tip, 3µl of annealing mix was added to each of the labelled eppendorf tubes. The eppendorf tubes were placed at 37°C for 15min, after which time 1.5µl of chase solution was added and further incubated at 37°C for 15min. The reaction was terminated by the addition of 4µl 90% formamide, 20mM EDTA, 0.3% bromophenol blue, 0.3% xylene cyanol. The samples were electrophoresed as described below.

Polyacrylamide gel electrophoresis

The sequence samples were electrophoresed through a 8% polyacrylamide 210x400x0.4mm vertical gel using a Sequi-Gen Nucleic Acid Sequencing Cell (Biorad). The gel was assembled and cast according to the manufacturer's instructions. The gel was pre-electrophoresed and preheated to 55°C, before addition of samples. The samples were denatured by heating at 80°C for 3mins before loading 2.5µl on to the gel. In order to minimise secondary structure, the samples were electrophoresed at 2,000 volts, to maintain temperature of the gel at 55°C. On completion of electrophoresis, the acrylamide gel was transferred to Whatmann 3MM and dried under vacuum at 80°C for 1 hr, before exposure to Kodak XAR-5 film with intensifying screens at RT for 1-3 days.

Computer programs used to analyse sequence

Sequence identity was investigated using a Wilbur-Lipman database search program, accessed through the Sequence programs available through the computer center at the Research School of Biological Sciences. The program compares a given sequence with all sequences in the databank using the algorithm of Wilbur and Lipman. The sequences are compared by alignment of sections of sequence. The highest alignment of base matches corresponds to the highest possible score, which is converted to a normalised score, providing an empirical guide for

evaluating the significance of similarity between the query and database sequences, (Wilbur and Lipman 1983).

Chapter 3

Characterisation of E1A-induced clones

Introduction

Differential screening of a subtractive cDNA library was used as a means of identifying cellular genes, induced during adenovirus infection. Results presented in this chapter show that a subset of cDNA inserts were isolated that were induced as a consequence of E1A expression during adenovirus infection. These clones were sequenced and have high sequence similarity to adeno-associated virus, the significance of which is discussed in chapter 4.

Differential screening of a subtracted library.

In order to isolate unknown cellular genes, influenced by the E1A region of adenovirus during infection, a subtractive cDNA library was constructed as described in Appendix 1. Cells were arrested in the G1 phase of the cell cycle by confluence before infection. cDNA was synthesized from poly A^+ RNA isolated from rat embryo fibroblasts 40 hrs post infection with WT adenovirus and hybridised to poly A^+ RNA isolated from cells infected with the E1A negative mutant dl 312, and adenoviral DNA . Single-stranded cDNA, presumably comprising of unique cellular sequences induced by E1A, was isolated by passage of the hybridisation mix through hydroxylapatite and cloned into the *Pst* 1 site of the pUC 9 plasmid. The resulting subtractive cDNA library, enriched for cellular sequences induced by E1A, comprised 22,000 clones.

Screening of the cDNA clones was necessary to eliminate any sequences viral in origin, or common to both of the original populations. Two different strategies were employed to identify E1A induced cellular sequences from the library. Approximately 11,000 clones were grown on nitrocellulose filters and processed as described in Appendix 1. Duplicate filters were probed with radiolabelled cDNA synthesized from mRNA of WT-infected cells (the E1A positive probe) or dl 312-infected cells (the E1A negative probe). Alternatively, duplicate filters of clones were probed with radiolabelled subtracted cDNA. The positive subtracted cDNA was synthesized from mRNA of WT-infected cells, hybridised to mRNA from dl 312 infected cells and

passaged through a hydroxylapatite column before use in filter hybridisation. The negative subtracted cDNA was synthesized from mRNA of dl 312-infected cells, hybridised to mRNA from WT- infected cells and eluted through a hydroxylapatite column before use in filter hybridisations.

The first screen of 11,000 clones with the E1A positive and E1A negative probes resulted in 612 clones hybridising strongly with the E1A positive probe and weakly or undetectably to the E1A negative probe. Out of 612 clones screened as positive, after the second round of screening, 250 clones were identified as strongly positive. 149 of these clones exhibiting no hybridisation to the negative probe and were stored at -70°C, while the remaining 101 clones weakly hybridised to the negative probe, and were subject to a third differential screen. After the final screen, 77 clones from 101 were deemed positively induced by E1A (demonstrated in fig.3.1a). These were screened with viral DNA to eliminate viral sequences and screened with the thymidine kinase gene, as thymidine kinase induction peaks at 36hrs after infection (Cheetham and Bellett 1982), and is E1A dependent (Braithwaite *et al.* 1983). No clones showed homology to adenovirus DNA or the thymidine kinase gene. The 77 clones were stored at -70°C.

A further 1,000 colonies from the subtracted library were screened using the subtracted cDNA probes. After the first screening, 144 clones were picked to be screened again. After screening the 144 clones with subtracted E1A-positive and E1A-negative probes, 18 clones hybridised to the subtracted positive probe and not to the negative probe, as shown in fig 3.1b. These clones showed no homology to adenoviral DNA nor the thymidine kinase gene when screened with the viral and cellular probe, and this chapter is devoted to the characterisation of these clones, as described below.

Thus a total of 244 clones were selected that appeared to represent putative cellular genes induced by E1A. These clones represented approximately 22% of the subtracted library.

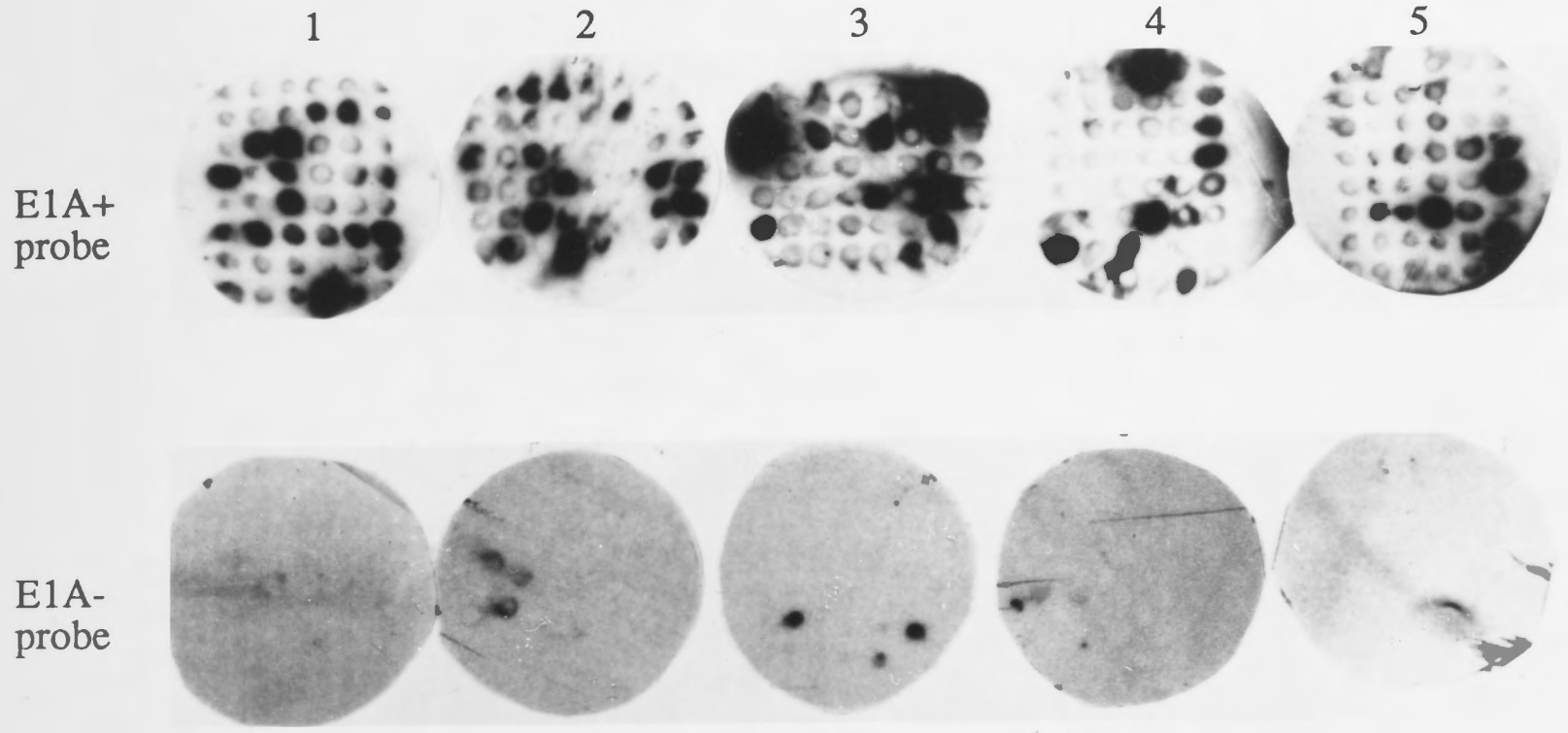


Figure 3.1.a 77 clones identified from 101 clones by differential screening

Duplicate filters were probed with radiolabelled cDNA synthesised from mRNA of WT-infected cells (the E1A+ probe), or from mRNA of dl 312-infected cells (the E1A- probe). After the third screening, 77 clones from 101 clones, hybridised uniquely to the E1A+ probe and not the E1A- probe.

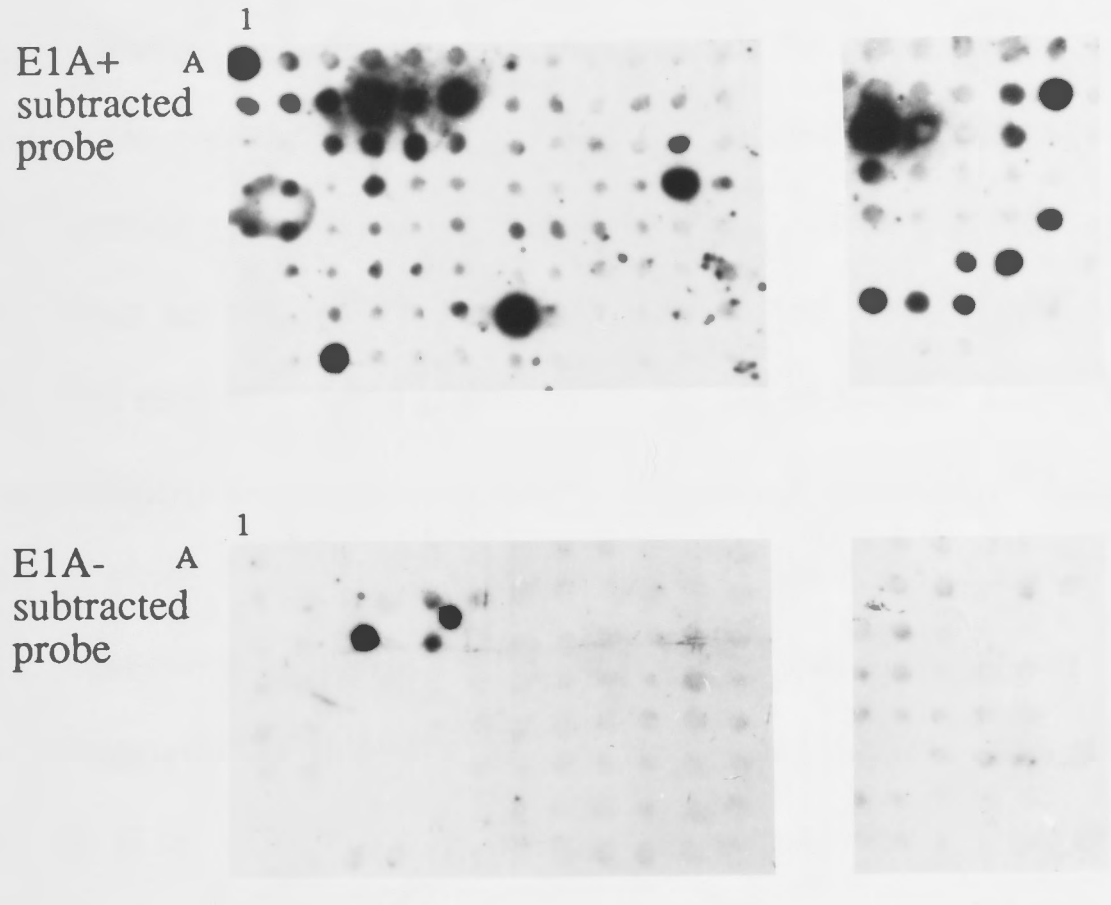


Figure 3.1.b. 18 clones identified from 144 clones after two rounds of differential screening, using subtractive cDNA probes.

Duplicate filters of clones were probed with radiolabelled subtracted cDNA. The E1A+ subtracted cDNA probe was synthesised from mRNA of WT-infected cells, hybridised to mRNA from dl 312 infected cells and passaged through a hydroxylapatite column before use in filter hybridisation. The E1A- subtracted cDNA probe was synthesised from mRNA of dl 312-infected cells, hybridised to mRNA from WT- infected cells and eluted through a hydroxylapatite column before use in filter hybridisations.

Characterisation of cellular sequences isolated by differential screening.

Initially the eighteen clones identified through two rounds of screening with subtracted probes (fig 3.1.b), were characterised through Northern analysis, Southern analysis and sequencing. The inserts of cDNAs within the plasmids ranged in size from 1.0kb to 0.13kb as shown in table 3.1. In order to identify duplications of insert, the clones were digested with *Pst*1 (to excise the inserts), electrophoresed, transferred to nylon membranes according to the method of Reed and Mann (1985), and hybridised with individual nick translated clones as explained in section 2.2.6., 2.2.7. Three clones from ten clones used as probes, showed sequence similarity. Clone 4 hybridised to the insert of clone 5 and clone 3, as shown in figure 3.2. Clones 1, 2, 6, 7, 8, 9 and 10 showed no cross hybridisation. This suggests that 7 clones out of 10 are unique and may potentially be different cellular sequences induced by the expression of E1A, although if the cDNA inserts are not full length, which is suggested from the small sizes of inserts, the clones could represent different areas of the same mRNA species.

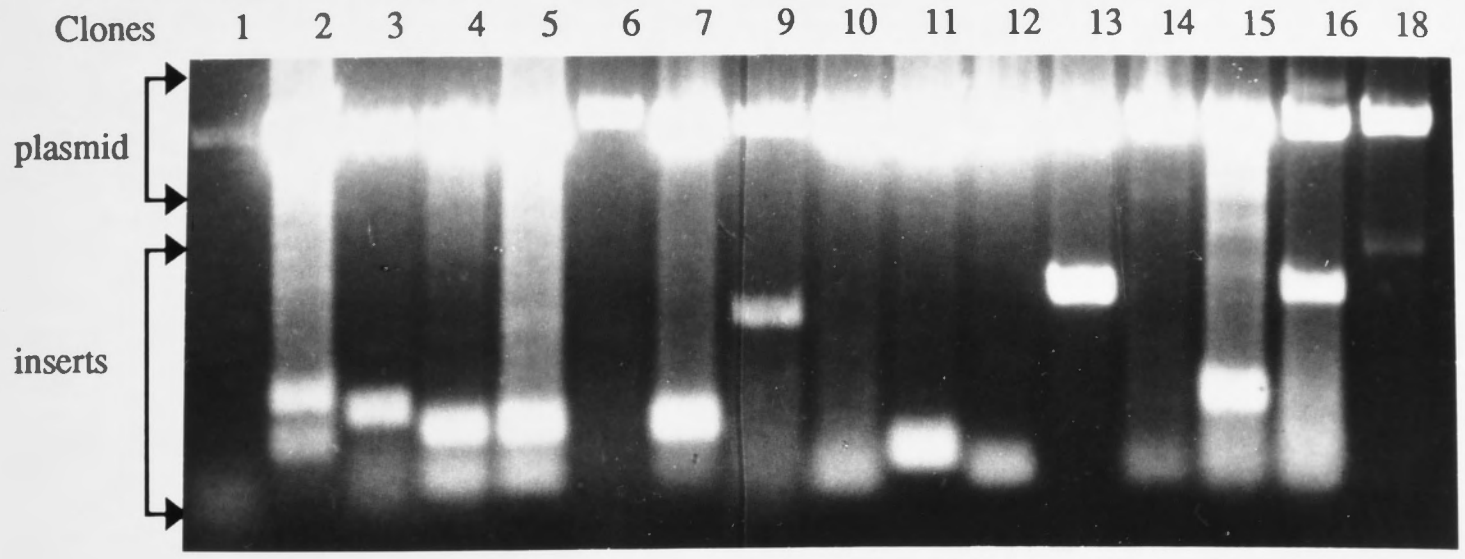
To investigate whether the clones were induced by E1A during adenovirus infection, Northern analysis was utilised. Poly A^+ RNA was isolated from WT-infected cells and dl 312 infected cells, glyoxylated and electrophoresed through agarose before transfer to nylon membranes, as described in 2.2.4. The time at which E1A transcription products were detectable was estimated by probing RNA, isolated from infected cells at different times after infection, with E1A. As shown in fig 3.3, at 25 infectious units per cell, E1A transcription was evident from 12-15hrs post infection and increased to a plateau at 30-40hrs pi. When 18 clones were used to probe poly A^+ RNA, isolated from WT infected cells and dl 312 infected cells 40 hrs after infection, 12 clones did not hybridise detectably to either RNA population, suggesting that the 12 clones were either not induced, or weakly induced beyond the sensitivity of Northern analysis (demonstrated in fig. 3.4). The remaining 6 clones hybridised strongly to discrete

Figure 3.2 Examples of cross hybridisation

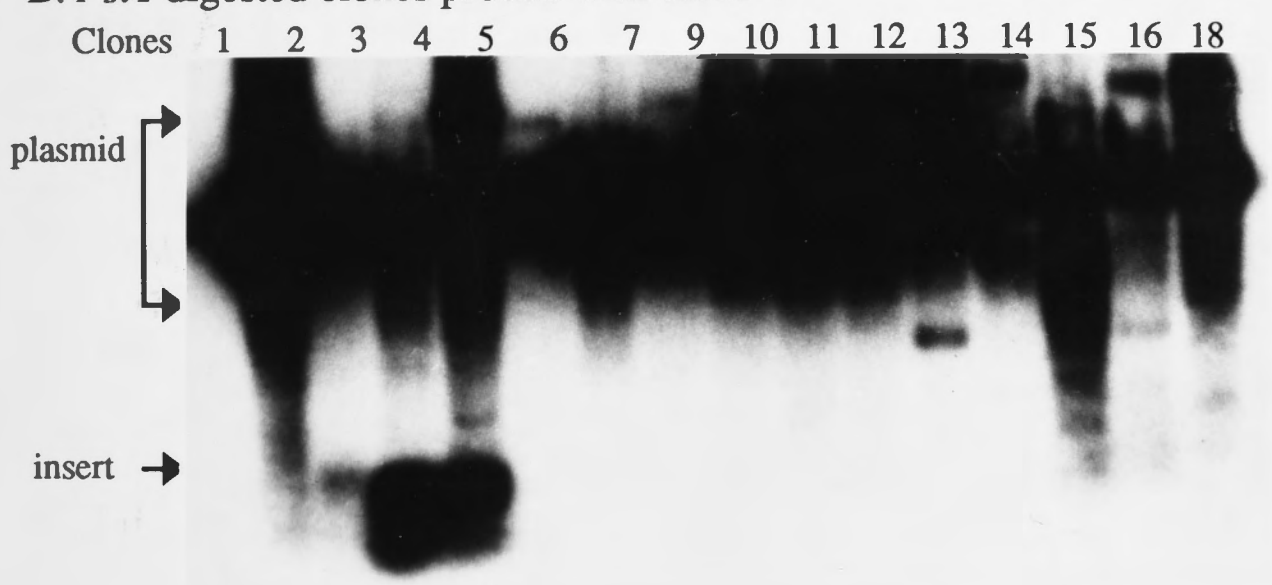
A) Clones were digested with *Pst* 1 (to excise the inserts), electrophoresed, transferred to nylon membranes and hybridised with individual nick translated probes. Three clones from ten clones used as probes, showed sequence similarity. B) Clone 4 hybridised to the inserts of clone 5 and clone 3. C) Clone 5 hybridised to the inserts of clone 4 and clone 3. D) Clones 1, 2, 6, 7, 8, 9 and 10 showed no cross hybridisation. A typical example is clone 7.

Figure 3.2. Cross hybridisation studies

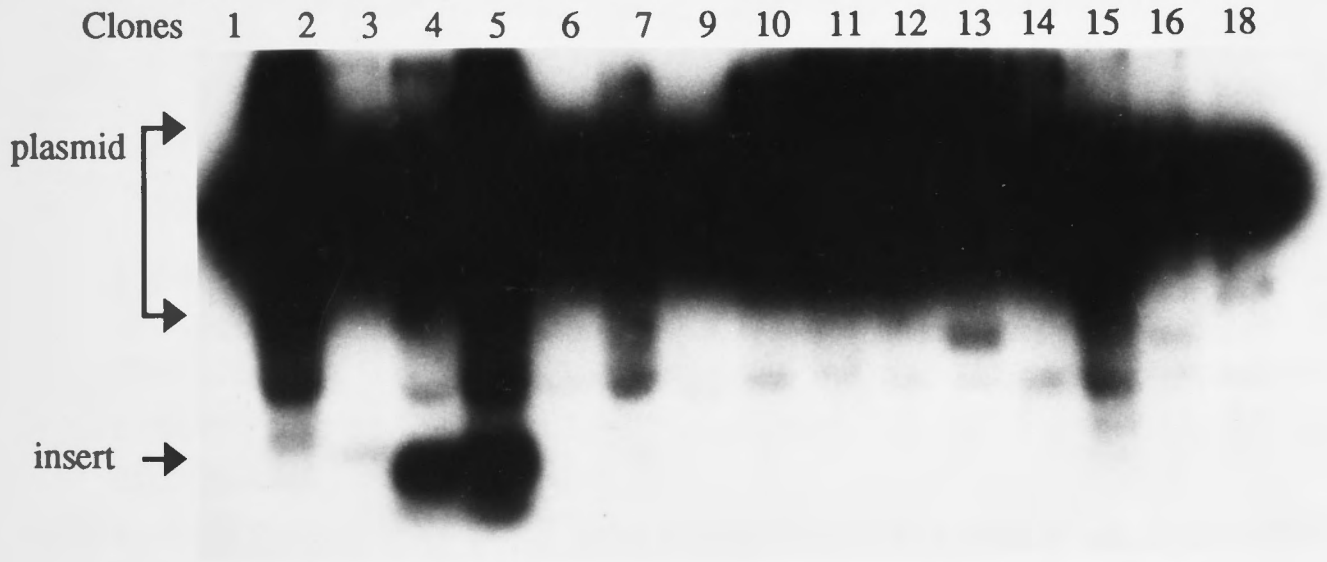
A. Ethidium Bromide stain of *Pst*1 digested clones



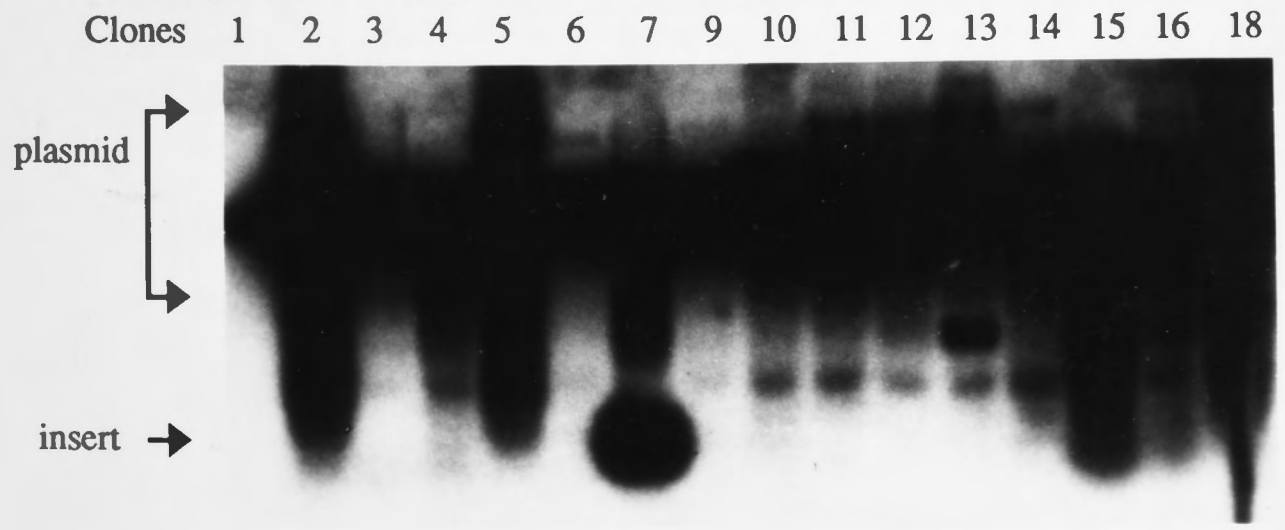
B. *Pst*1 digested clones probed with clone 4



C. *Pst*1 digested clones probed with clone 5



D. *Pst*1 digested clones probed with clone 7



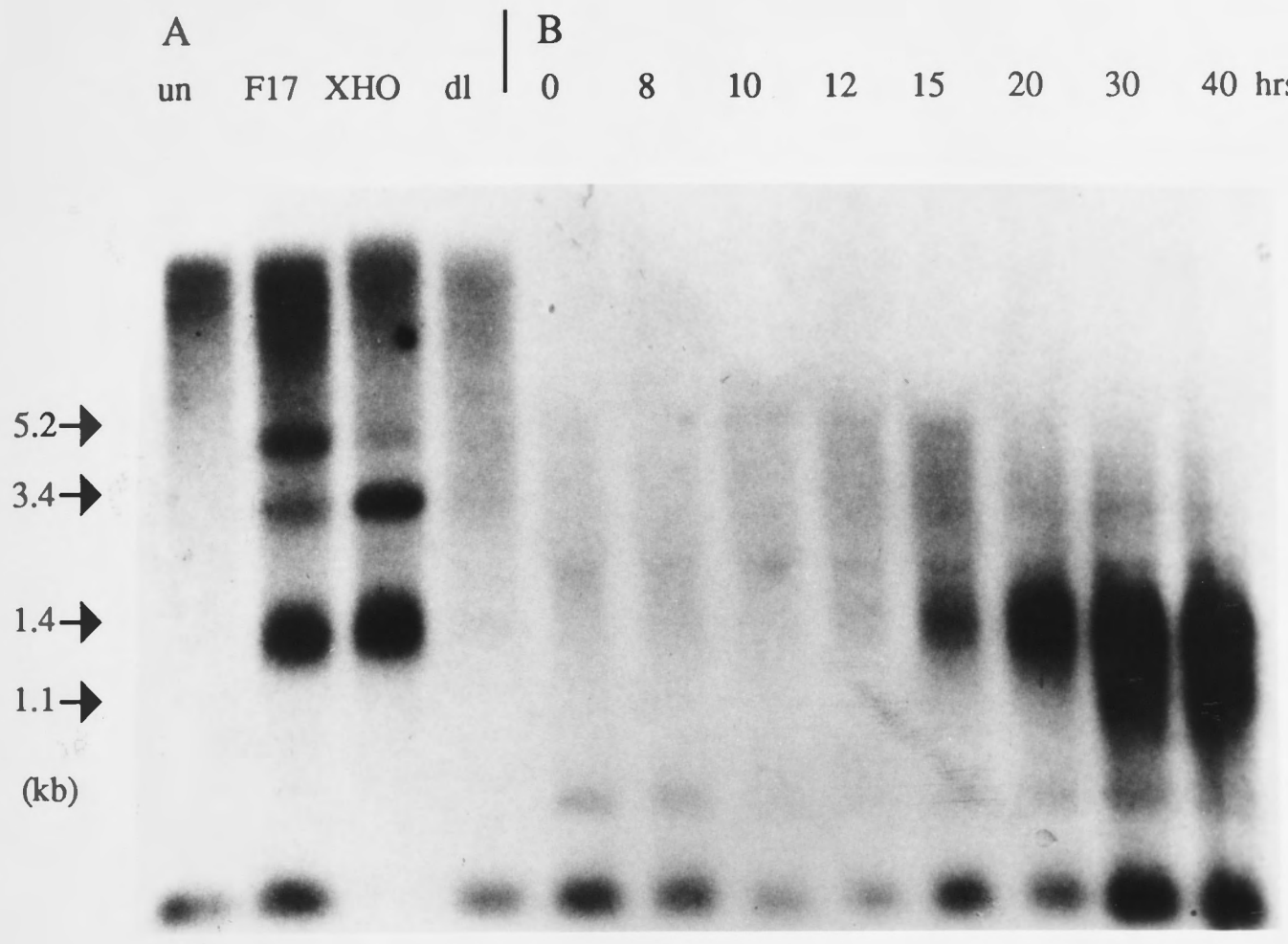


Figure 3.3 E1A transcription during adenovirus infection of semipermisive cells

The time at which E1A transcription products were detectable was estimated by probing RNA, isolated from infected REFs at different times after infection, with E1A.

A. 5µg poly A+ RNA isolated from uninfected (un) cells, Ad-transformed cell lines F17 and XHO-BRK, and dl 312 infected (dl) cells.

B. 20µg total cytoplasmic RNA isolated from cells infected with WT at various times aftr infection. E1A transcription was evident from 12-15hrs post infection at 25 infectious units per cell.

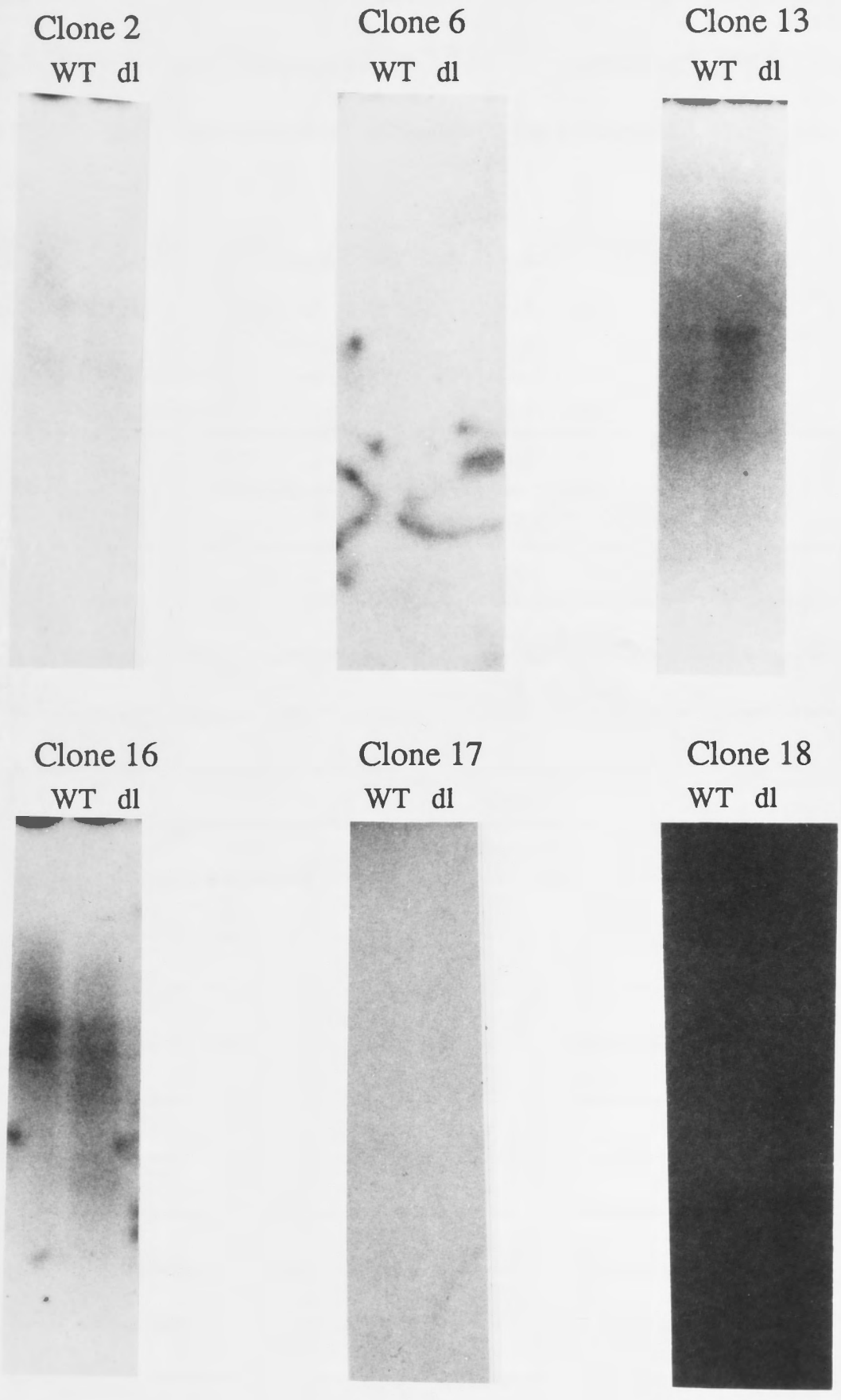


Figure 3.4 Examples of cDNA clones weakly induced by E1A.
18 clones were used to probe 5µg poly A+ RNA, isolated from WT (WT) infected cells and dl 312 (dl) infected cells, 40 hrs after infection. 12 clones did not hybridise differentially to either RNA population, suggesting that the 12 clones were not induced, or weakly induced beyond the sensitivity of Northern analysis.
To ensure equal RNA loading, an RNA gel was stained with acridine orange and some membranes that were probed with the uninduced clones, were reprobed with induced clones

RNA species from WT infected cells and not to dl 312 infected cells, as shown in table 3.2 and figure 3.5. This result implies that 6 of 18 clones are positively influenced by E1A, during adenovirus infection, while 12 of 18 clones are not induced by E1A or alternatively are in sufficiently low abundance to be undetectable by Northern analysis.

Table 3.1. Sizes of inserts of 18 E1A-induced clones isolated by differential screening of a subtracted library using subtracted probes.

Clone number	Size of insert (Kb)
1	0.540
2	0.330,0.230
3	0.320
4	0.450, 0.240
5	0.475, 0.240
6[A]	0.0
7	0.470
8	0.500
9	0.565
10	0.135
11	0.180
12	0.130
13	0.925
14[A]	0.0
15	0.498
16	0.670
17[A]	0.0
18	0.940

Sizes of inserts were estimated using a fragment length analysis system computer program, adapted from Duggelby *et al.* (1981)

[A] -Clones with undetectable inserts (≤100bp) were discarded

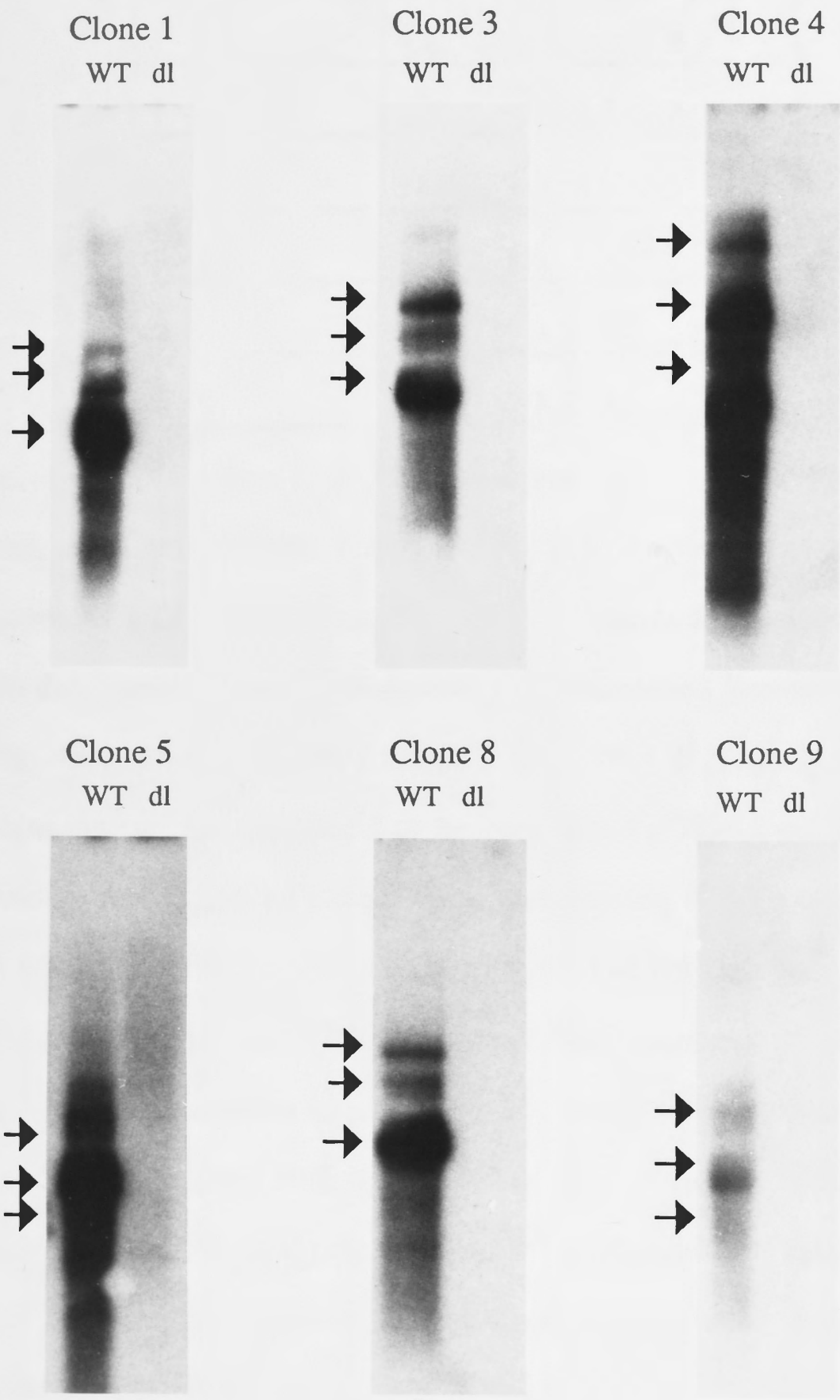


Figure 3.5 Examples of cDNA clones strongly induced by E1A.
18 clones were used to probe 5µg poly A+ RNA, isolated from WT (WT) infected cells and dl 312 (dl) infected cells, 40 hrs after infection. 6 clones hybridised strongly to discrete RNA species from WT infected cells and not to RNA from dl 312 infected cells.

Table 3.2 Size of mRNA species complementary to 6 clones induced by E1A

Clone	RNA (kb)
1	4.8, 3.6, 3.3, 2.6, 2.3
3	5, 3.6, 3.3, 2.6, 2.3
4	5, 3.9, 3.6, 2.6, 2.3
5	5, 4.2, 3.6, 3.3, 2.6
8	4.2, 3.6, 3.3, 2.6, 2.3
9	3.9, 3.6, 3.3, 2.6, 2.3

Identification of cDNA inserts by sequence analysis

To establish the identity of the cDNA inserts as known or previously undescribed cellular genes, it was necessary to sequence the inserts and compare sequences to established data banks. The cDNA inserts were subcloned into pGEM1 for plasmid sequencing. Inserts were excised from pUC 9 by *Pst* 1 digestion, isolated from low melting point agarose and religated into the *Pst*1 site of pGEM1, (section 2.2.8, 2.2.9). The plasmids were sequenced according to the GemSeq K/RT system (Promega) as described in 2.2.10. Partial sequences are shown in fig 3.6a and 3.6b. Sequence identity was investigated using a Wilbur-Lipman database search program, as described in section 2.2.9. Five random sequences of 100 nucleotides length, comprising 20% adenosine, 20% thymine, 30% cytosine and 30% guanine were generated and compared to the databank, using the Wilbur Lipman algorithm to establish an arbitrary score to use as a guide for evaluating alignment significance. Sequence similarity which resulted in a score of ≤25 was considered to be of no significance.

Sequence analysis of poorly induced clones

Initially a number of poorly induced clones, from the 18 subtracted clones under investigation, were sequenced and identified. Of the four poorly induced clones that

Figure 3.6a Partial sequences of weakly induced clones

Sequence of clone 2, 330bp fragment

```
                                                                    50
TCTTGCATTC  AATTGACGCA  TCCGCGCTTC  AAACTGACCA  CGCATTGCCG
                                                                   100
TGCCTTGCAC  GAGGGCAGAC  ATGTCAAGTC  GGATAATCTC  TTTTGACTGC
                                                                   150
AATTTTTCAG  GGACTTTATT  GTGCCAATTG  CTTGGGCGAG  TCTCACACGC
                                                                   200
ATGTTTACAC  ACTGGGTACG  ACGATTTAAT  ATCTCGATGG  TGCGGGCGAT
                                                                   250
TTCGTCTCAC  GACGTATGAT  CTACTTACGT  ACCGGCTTGT  TGTGTTAAGT

TAATTCAATT  CGTTAGGTAT  GTTGGGCCGA  CCTTAGTTTA  TCC
```

Clone 2, 270bp insert

```
                                                                    50
AGTAAGCTAA  TTTGTAGGCA  TCTGGTCTTA  CTAAGCAGAG  CCCTATCATC
                                                                   100
TCTCTCTAAG  ACTCCGTCGA  AGTCGTTTGG  TTGGACGGAA  GCCTTCTATG
                                                                   150
TGTCTGGTAC  GCGTCCCGCA  TTGGAAATGG  TCAGCGAACC  AATCAGCAGG
                                                                   200
GTCATCGCTA  GCCAGATCTT  ACGCGGAGAT  GTGGCCGGAT  ACGGGTCCAG

ATGCGGTTGC  TCGGCTATAT  ATA
```

Clone 11, 180bp insert

```
                                                                    50
TACAATTACA  TAAGGTTATT  ATCAACATAC  ATTTATAGAC  TATGAAGCAG
                                                                   100
GGCGGTGGGC  GGGCGGCGGG  CCGGGACGCT  GGCGGTCGCC  ACCCGGCTTT
                                                                   150
CTCCTAGCGC  CGGCGTGGTA  GATGAGAAAT  TCACGGAAGA  AAGCATCAAC

GAGCAAATTC  GTGCCTTGAG  CACTATAATG  GCCCCCCCCC
```

Clone 12, 130bp insert

```
                                                                    50
GGGCGGTGGG  CGGGCGGCGG  GCCGGGACGC  TGGCGGTCGC  ACCCGGCTTC
                                                                   100
TCCTAGCGCC  GGCTGGTAGA  TGAGAAATTC  ACGGAAGAAA  GCATCAACGA

GCAAATTCGT  GCCTTGAGCA  CTATAATGGC  CCCCCCCCCC
```

Figure 3.6b Partial sequence of E1A induced clones

___-Homology to AAV sequences

Clone 1, 530bp insert

```
                                                          50
AGCAGGCCCG  GGGCGGTCAT  GTCTCTTTCC  TAGTGTCGCG  TCGCCCCCCG
                                                         100
GGGGGGGTTC  CGGCGGGCGC  CCAGCGTTCG  GGGCTTCAAG  TGGAACCACA
                                                         150
CGCAGTCAAG  GCTTCATTTT  CTCAGGCGGA  GCGAGTGACA  TTGGGACAGT

CTAGGAATGC  TTCTGACTGT  TACGCAGACG  AGTATAAAGA  ATT......
                                                         150
..........  CTTATCTATC  CAACCCTAA   AGCAACAGAC  AAGCAGCATC
                                                         100
CAGAGTCTAA  CACACAAGCG  CTTTTCCAGC  AGTCTGTGCA  GGACAGAGAT
                                                          50
GTGTACCTTC  AGGGCGCCCA  TCTGGGCAAA  GATTCCACGA  CACGGACGGG
                                                           0
AGCAGGCAGT  CGCGCCCAGG  GCCCCGCCGT  CCGCCCCCCC  CGACGACCGA
```

Clone 3, 330bp insert

```
                                                          50
TGGTCTGGCA  GGACAGAGAT  GTGTACCTTC  AGGGGCCCAT  CTGGGCAAAG
                                                         100
ATTCCACACA  CGGACGGACA  TTTTCACCCC  TTCCTCATGG  GTGGATTCGG
                                                         150
.........AA CATCTATCAT  AGAATACCCG  TACCTGCGAA  TCCTTCGACC
                                                         100
ACCTTAGTGC  TGGCAGAACG  GTTTGCTGTC  CTTCGATCAC  TACCAGCTAC
                                                          50
TCCCACGCGC  GACAGGTCAG  CGTGGTAGAT  CGAGTCGTAC  GCGGCCTTCG
                                                           0
CTCGCTCGGG  TGACCCGCAC  TCAGCGTCCC  CTGATCCCCG  AGAGACACCC
```

Clone 4, 239bp insert

```
                                                          50
GGGGTGTCGA  GCACCTTTAG  TGCGGCAAAG  TTTGCTTCCT  TCATCACACA
                                                         100
GTAGCTCCAC  GGGACAGGTC  AGCTGGGGAA  TCGGATGGGG  ACTGCA
```

Clone 8, 170bp insert

```
                                                          50
GCTAGGGGGG  CGACCTTCGA  CCACCTTCAG  TGACGGCAAA  GTTTTGCTTC

CTTCTGCAGG  TCGACT
```

were sequenced, the two inserts isolated from clone 2 showed no significant similarity to sequences in EMBL (release 19) and GENBANK (release 47) databases. Clones 11 and 12 showed remarkable similarity (scores 59 and 65 respectively) over a subsequence of 64 nucleotides to a yeast nuclear gene necessary for the synthesis of cytochrome b, designated CBP6 (Dieckmann and Tzagoloff 1985, EMBL no. 19439), as shown in figure 3.7. The region of homology in CBP6 extended from nucleotide 572 to nucleotide 637. The predicted translation product of the conserved region contains 18 hydrophilic amino acids similar to amino acids from position 187 to 204 of the CBP6 protein. Using the Chou-Fasman algorithm, it was possible to predict the secondary structure of the conserved region as an α helix, containing no obvious regulatory features (Chou and Fasman, 1978). Any secondary structure predictions, however, must be considered with caution, as most prediction methods have an approximate 55% level of accuracy in prediction of the conformation state of amino acid residues (Nishikawa 1983, Kabsch and Sander 1983).

Sequence analysis of induced clones

The sequences of four strongly induced clones were determined and analysed. After comparison with sequences in GENBANK and EMBL, sequences from clone 1, clone 3, clone 4 (239bp insert) and clone 8 showed significant similarity (scores 70, 78, 63, 30, respectively) to regions within the second open reading frame of adeno-associated virus type 2 (AAV 2), specifically within the genes encoding structural coat proteins between map units 75.8 and 91, as shown in fig.3.8.

Figure 3.7 Alignment of Yeast (S.cerevisiae) nuclear gene CBP6 for cytochrome b, (937 bases) with clone 11 (181 bases)
: -nucleotide homology

```
X         10         20         30         40         50         60
ATACGATTATTTTGGAAGTTTATAAAAGAAGTGCGGAAATCACATCTGCTGTTTATTTAGCCATTCCTCA

          80         90        100        110        120        130
CACTAATAGTTAAAGTACTTTCATAGCAGCTCTGCGCATGGTCGGACATGCGAAAAATTCTGATATCAAG

         150        160        170        180        190        200
AAAAAGCGAAATATTTCCGGCCTTGTAGGGGCCAAAACATTAACGTATATCAAGATTTCCTGTGGTAGCA

         220        230        240        250        260        270
ACATTATAAGAAAAAAAGGTAGCCTTCATTGAAACATTCTCTCTATCAGCTTACCAAGTTAAACTCCGTA

         290        300        310        320        330        340
TTCCACAAGCAAGTGCCAAAATGTCTTCTTCCCAGGTCGTCAGGGATTCTGCCAAAAAATTAGTTAATTT

         360        370        380        390        400        410
ACTGGAAAAATATCCAAAGGATCGTATACACCACTTGGTCTCATTCAGGGATGTACAAATAGCAAGATTT

         430        440        450        460        470        480
AGACGTGTAGCGGGTCTGCCAAATGTAGATGACAAAGGAAAATCTATAAAAGAGAAAAAACCCTCATTAG  CBP6
                                    :: :  ::::       :  : : :   :
                                   TACAATTACATAAGGTTATTATCAACATACATTT  Clone 11
                                   X        10        20        30

         500        510        520        530        540        550
ATGAAATAAAAAGTATAATTAACAGAACTTCCGGTCCATTAGGACTGAATAAGGAGATGTTAACCAAAAT  CBP6
::   : ::   ::: :         :          :  :     :::       :  :
ATAGACTATGAAGCAGGGCGGTGGGCGGGCGGCGGGCCGGGACGCTGGCGGTCGCCACCCGGCTTTCTCC  Clone 11
     40         50         60         70         80         90        100

         570        580        590        600        610        620
TCAAAATAAAATGGTAGATGAGAAATTCACGGAAGAAAGCATCAACGAGCAAATTCGTGCCTTGAGCACT  CBP6
:          ::::::::::::::::::::::::::::::::::::::::::::::::::::::::::: :
TAGCGCCGGCGTGGTAGATGAGAAATTCACGGAAGAAAGCATCAACGAGCAAATTCGTGCCTTGAGCA-T  Clone 11
     110        120        130        140        150        160        170

         640        650        660        670        680        690
ATAATGAATAATAAATTCAGAAACTATTACGATATTGGCGATAAGCTCTATAAACCTGCAGGAAATCCCC  CBP6
::::::
ATAATGGC                                                                 Clone 11
       X

         710        720        730        740        750        760
AATATTATCAACGGTTAATAAATGCCGTTGACGGTAAGAAAAAGGAAAGCTTATTTACTGCAATGAGAAC

         780        790        800        810        820        830
TGTATTATTTGGTAAATAAAGAGCACATTATTTTCTAAGCTTGTAAATACATATTTATTCATAATGGAGA

         850        860        870        880        890        900
ACGTTATTCAAATTTATCTGTGAATTTCTTTACTCGAGGTATACTTCCGCAAAGGAAATTCTACTTAGCA

         920        930    X
AATCCTATGGTAACGTCATTGTTTTGT
```

Figure 3.8 Alignment of induced clones with AAV 2
SP6 - sequenced from the sp6 promoter of pGEM
T7 - sequenced from the T7 promoter of pGEM, converted to antisense complementary sequence
: - nucleotide homology

```
         3510       3520       3530       3540       3550       3560
TCATGAATCCTCTCATCGACCAGTACCTGTATTACTTGAGCAGAACAAACACTCCAAGTGGAACCACCAC  AAV 2
                                                    :::::::::::::: ::
                                                    CAAGTGGAACCAC-AC  clone 1 (SP6)
                                                       90

         3580       3590       3600       3610       3620       3630
GCAGTCAAGGCTTCAGTTTTCTCAGGCCGGAGCGAGTGACATTCGGGACCAGTCTAGGAACTGGCTTCCT  AAV 2
:::::::::::::::: :::::::::::: ::::::::::::::: ::   :::::::::::   :::::
GCAGTCAAGGCTTCA-TTTTCTCAGGC-GGAGCGAGTGACATTGGGA--CAGTCTAGGAAT--GCTTCTG  clone 1 (SP6)
        110        120        130        140        150         160

         3650       3660       3670       3680       3690       3700
GGACCCTGTTACCGCCAGCAGCGAGTATCAAAGACATCTGCGGATAACAACAACAGTGAATACTCGTGGA  AAV 2
     :::::::    ::::       :::::  :
A----CTGTTAC-----GCAGACGAGTATAAAGAATT                                    clone 1 (SP6)
        170            180       190X

         3720       3730       3740       3750       3760       3770
CTGGAGCTACCAAGTACCACCTCAATGGCAGAGACTCTCTGGTGAATCCGGCCATGGCAAGCCACAAGGA



         3790       3800       3810       3820       3830       3840
CGATGAAGAAAAGTTTTTTCCTCAGAGCGGGGTTCTCATCTTTGGGAAGCAAGGCTCAGAGAAAACAAAT



         3860       3870       3880       3890       3900       3910
GTGAACATTGAAAAGGTCATGATTACAGACGAAGAGGAAATCGGAACAACCAATCCCGTGGCTACGGAGC



         3930       3940       3950       3960       3970       3980
AGTATGGTTCTGTATCTACCAACCTCCAGAGAGGCAACAGACAAGCAGCTACCGCAGATGTCAACACACA  AAV 2
            ::::::::::::   :     ::::::::::::::::  :  ::::    ::::::::
          CTTATCTACCAACCCTAAA----GCAACAGACAAGCAGCATC--CAGAGTCTAACACACA  clone 1 (T7)
          X       180        170            160         150        140

         4000       4010       4020       4030       4040       4050
AGGCGTTCTTCCAGGCATGGTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCATCTGGGCAAAGATT  AAV 2
::    :: ::::::     ::::::::::::::::::::::::::::::::::::::::::::::::::
AGCGCTT-TTCCAGCA---GTCTGGCAGGACAGAGATGTGTACCTTCAGGGGCCCATCTGGGCAAAGATT  clone 1 (T7)
    130         120          110        100        90         80         70
                  ::::::::::::::::::     :::::::::::::: ::::::::::::
                  TGGTCTGGCAGGACAGAGATTGTA--CTTCAGGGGCCCAT-TGGGCAAAGATT  clone 3
                  X         10         20          30          40

         4070       4080       4090       4100       4110       4120
CCACACACGGACGGACATTTTCACCCCTCTCCCCTCATGGGTGGATTCGGACTTAAACACCCTCCTCCAC  AAV 2
::::::::::::::
CCACACACGGACGG                                                           clone 1 (T7)
        60
:::::::::::::::::::::::::::::    ::::::::::::::::::
CCACACACGGACGGACATTTTCACCCCTT---CCTCATGGGTGGATTCGG                       clone 3
         60         70           80         90     X
```

```
        4140      4150      4160      4170      4180      4190
AGATTCTCATCAAGAACACCCCGGTACCTGCGAATCCTTCGACCACCTTCAGTGCGGCAAAGTTTGCTTC  AAV 2
                                    :::: :::::: ::::::::::::::::::::
                                    TCGAGCACCTTTAGTGCGGCAAAGTTTGCTTC  clone 4
                                       10        20        30
                                    ::::::::::::::::::::::::::::::::::
                                    TCGACCACCTTCAGTGCGGCAAAGTTTGCTTC  clone 8
                                      20        30        40

        4210      4220      4230      4240      4250      4260
CTTCATCACACAGTACTCCACGGGACACGGTCAGCGTGGAGATCGAGTGGGAGCTGCAGAAGGAAAACAG  AAV 2
::::::::::::::: :::::::::::::::::::  ::  ::::  ::::  ::::::
CTTCATCACACAGTACTCCACGGGACACGGTCAGCTGGGGAATCGGATGGGGACTGCAG              clone 4
40         50        60         70        80        90      X
::::  ::    :
CTTCTGCAGGTCGACT                                                         clone 8
 50        60    X
```

Terminator

```
        4280      4290      4300      4310      4320      4330
CAAACGCTGGAATCCCGAAATTCAGTACACTTCCAACTACAACAAGTCTGTTAATCGTGGACTTACCGTG

        4350      4360      4370      4380      4390      4400
GATACTAATGGCGTGTATTCAGAGCCTCGCCCCATTGGCACCAGATACCTGACTCGTAATCTGTAATTGC
```

Polyadenylation signal

```
        4420      4430      4440      4450      4460      4470
TTGTTAATCAATAAACCGTTTAATTCGTTTCAGTTGAACTTTGGTCTCTGCGTATTTCTTTCTTATCTAG

        4490      4500      4510      4520      4530      4540
TTTCCATGGCTACGTAGATAAGTAGCATGGCGGGTTAATCATTAACTACAAGGAACCCCTAGTGATGGAG
```

Inverted terminal repeat

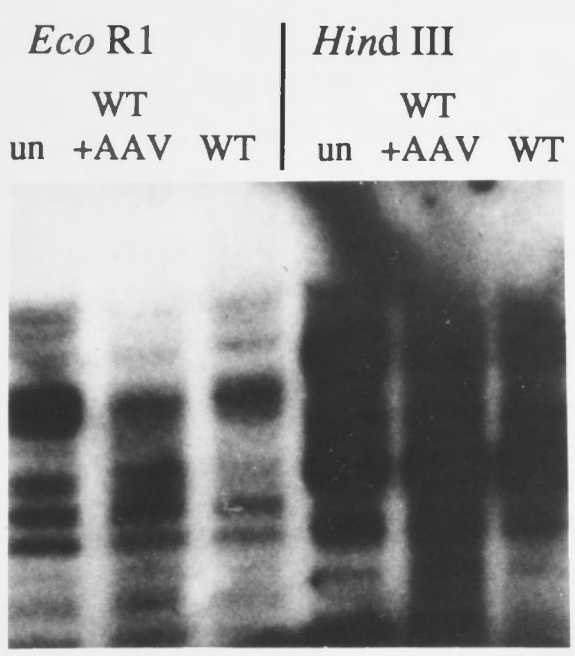
```
        4560      4570      4580      4590      4600      4610
TTGGCCACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGGGCGACCAAAGGTCGCCCGACGCCCGG

        4630      4640      4650      4660      4670 X
GCTTTGCCCGGGCGGCCTCAGTGAGCGAGCGAGCGCGCAGAGAGGGAGTGGCCAA-3'
```

Southern analysis of clones 3, 5, and 11

Clones 3, 5 and 11 were analyzed at the genomic level by Southern analysis. Uninfected rat DNA and rat DNA isolated 40hrs pi with WT or WT and AAV, were digested with *Eco*R1 and *Hind*III, electrophoresed through agarose and transferred to nylon membranes as described in section 2.2.6. The membranes were probed with α-tubulin, clone 3, clone 5 and clone 11. There are approximately 14 copies of the α-tubulin gene in the rat genome (Lemischka and Sharp 1982). Thus, by comparison of densitometer readings between Southerns probed with α-tubulin and clone 11 (fig 3.9), it was possible to estimate one gene copy of clone 11 per cellular genome. Although clone 11 only hybridised to a single band within the *Hind*III-digested DNA, and not to any *Eco*R1-digested DNA, the result is unlikely to be an artifact, because of the highly stringent conditions of the experiment. Perhaps the homologous EcoR1-generated fragments migrated from the agarose gel into the buffer during electrophoresis.

When DNA was probed by clones 3 and 5 (fig 3.10) there was strong hybridisation to the smear of DNA isolated from cells infected with WT and AAV, reflecting the type of hybridisation observed when AAV genomic DNA was used to probe the DNA. Through sequence analysis, clone 3 was identified as part of the AAV genome, and the Southern results imply that clone 5 also contained AAV sequences. Cross-hybridisation studies showed that clone 5 cross-hybridised to clone 4 (fig 3.2). Clone 4 was identified as containing AAV sequences, thus it is likely that clone 5 contained a similar region of AAV sequences. Hybridisation of clone 3 and clone 5 to cellular sequences in uninfected and WT infected DNA was suggestive of a cellular subsequence within clone 3 and clone 5, unrelated to AAV sequences. Indeed, 60 nucleotides present at the terminus of clone 3 showed no significant similarity to AAV, or to any genes within the database, and thus are likely to represent an undescribed cellular mRNA.

A.



B.

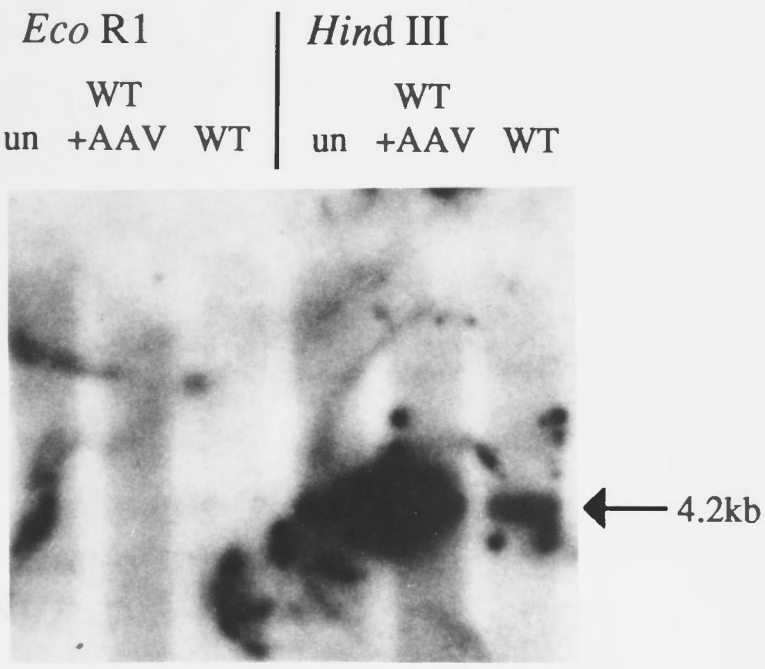


Figure 3.9 Southern analysis of an uninduced clone.
20μg of uninfected rat DNA (un) and rat DNA isolated 40hrs pi with WT or WT+AAV, were digested with *Eco*R1 or *Hin*dIII, electrophoresed through agarose and transferred to nylon membranes. The membranes were probed with A. α-tubulin and B. clone 11. There are approximately 14 copies of the α-tubulin gene. Comparison of relative autoradiograph exposures suggested 1 copy of clone 11 gene per cell.

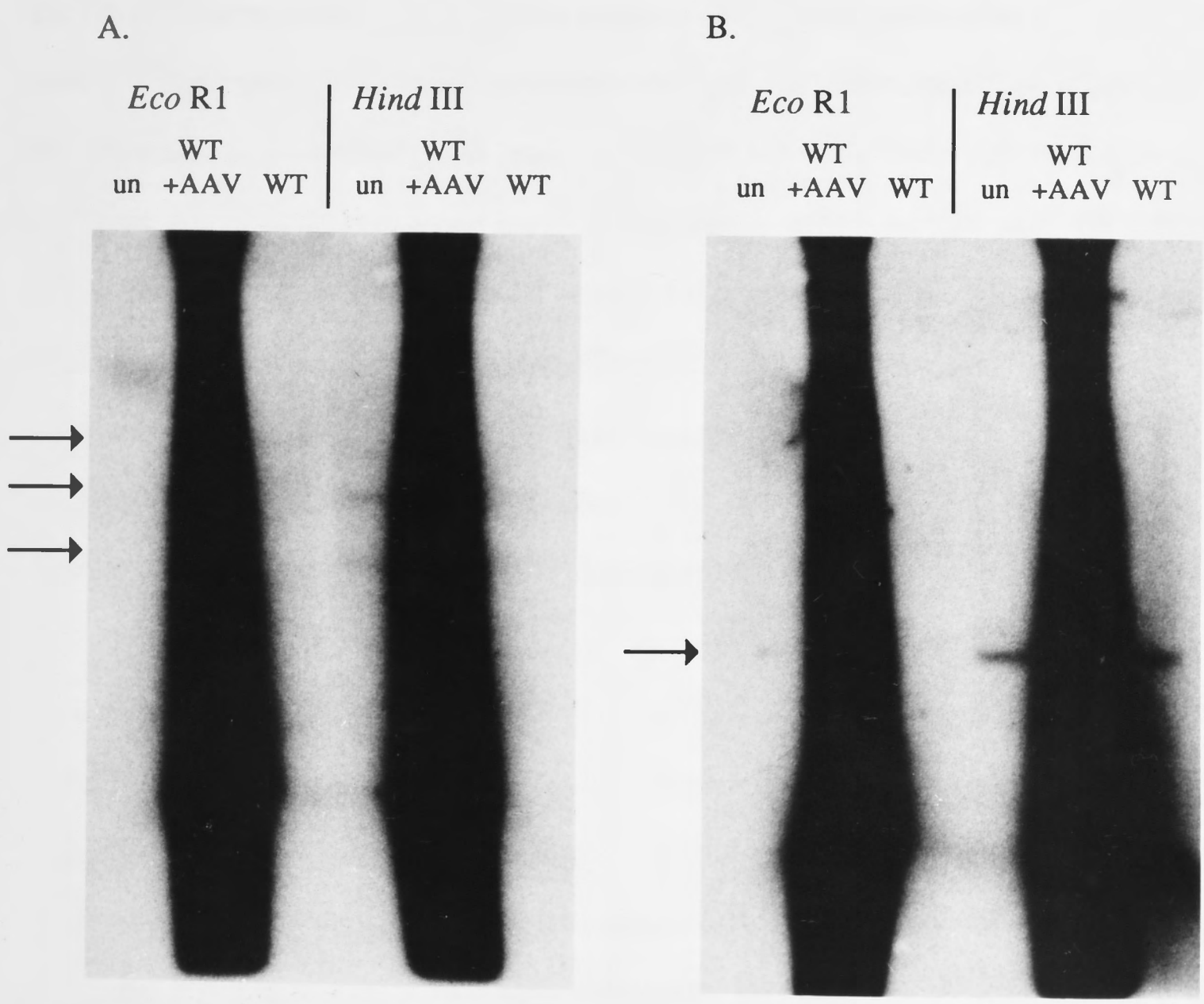


Figure 3.10. Southern analysis of induced clones.
20μg of uninfected rat DNA (un) and rat DNA isolated 40hrs pi with WT or WT+AAV, were digested with *Eco*R1 or *Hin*dIII, electrophoresed through agarose and transferred to nylon membranes. The membranes were probed with A) clone 3 and B) clone 5.

Discussion

Subtractive hybridisation was the principal component in the construction of a subtractive library, enabling the enrichment of low abundance mRNA species, represented as cDNA in the cDNA library of 22,000 clones. The principal interest in the library was to isolate cDNA clones representing mRNA induced by E1A, that may have an important role in transactivating other cellular genes, resulting ultimately in transformation. The library was constructed from rat embryo fibroblasts 40 hrs after infection, allowing the maximum level of activation of cellular genes by the E1A region of adenovirus. This assumption was based on the kinetics of induction of the cellular gene, thymidine kinase, by adenovirus. Thymidine kinase levels of cells arrested in the G1 phase of the cell cycle (by confluence) increase 2-5 fold after adenovirus infection, peaking at 36hrs (Cheetham and Bellett 1982, Liu *et al.* 1985). This increase is dependent on the expression of viral E1A (Braithwaite *et al.* 1983).

By eliminating cDNA sequences representing mRNA sequences common to the WT infected cell population and the dl 312 (E1A negative) infected cell population through hybridisation, the library was enriched for cDNA clones induced by E1A. Included among these clones would be those representing cellular genes known to be induced by E1A, eg. thymidine kinase, 70-kDa heat shock protein, PCNA (proliferating cell nuclear antigen, also known as cyclin), thymidylate synthetase, β-tubulin and others (as discussed in section 1.8.1.). As the subtractive technique is inefficient, the library would also contain clones representing cellular mRNA common to both WT infected and dl 312 infected cell populations. The cDNA used to make the library, was hybridised with an excess of adenoviral DNA, to eliminate common viral sequences. From 12,000 clones differentially screened, 244 clones were selected in total, that appeared to represent cellular genes induced by E1A. From 11,000 clones, after the second round of screening, 250 clones were identified as strongly positive. 149 of these clones exhibiting no hybridisation to the negative probe and were stored at -70°C.

It was thought at that time that these 149 clones were more likely to represent viral transcripts, and less likely to represent cellular regulatory transcripts induced from a basal level, as they did not hybridise to the negative probe. Thus, the remaining 101 clones that weakly hybridised to the negative probe, were subject to a third differential screen. After the final screen, 77 clones from 101 were deemed positively induced by E1A. Eighteen clones, from 1,000 clones, were identified through two rounds of screening with subtracted cDNA probes. In retrospect, all 244 clones (149+77+18 clones) should have been finally tested with both screening regimes to eliminate false positives, generated by the different screening methods. All of these clones were screened with adenovirus DNA, to identify viral sequences and screened for thymidine kinase sequences. All clones were negative for viral sequences, and for thymidine kinase sequences. As mentioned above, thymidine kinase is induced during adenovirus infection and the lack of thymidine kinase sequences amongst the clones was unexpected. The lack of hybridisation to thymidine kinase suggests the subtractive hybridisation technique does not detect small changes in common transcripts, or perhaps an aberrant infection due to the presence of AAV, which will be discussed in chapter 4. Only eighteen clones, identified through two rounds of screening with subtracted cDNA probes, were investigated further, by Northern and Southern analysis.

To confirm that the clones were induced by E1A during infection, 18 clones were used to probe nylon membranes containing poly A^+ RNA prepared from cells 40 hrs after infection with WT or dl 312. 6 of 18 clones were highly induced by E1A, 40 hours after infection, as suggested by strong hybridisation to discrete RNA bands from WT-infected cells and not dl 312-infected cells. These induced mRNAs may not be a primary consequence of E1A activation. E1A proteins are detected within infected cells at approximately 7-13 hrs after infection at 100iu per cell (Jackson and Bellett 1989), increasing to a maximum level at 20hrs after infection (Jackson and Bellett 1987). At

25iu per cell, it was possible to detect E1A mRNA species at 12-15 hrs, peaking at 40hrs after infection. As the library was constructed relatively late in infection, after the start of viral DNA replication, the clones may represent mRNA indirectly influenced by E1A. 12 of 18 clones exhibited no differential hybridisation to RNA isolated from WT-infected cells or dl 312 infected cells 40hrs pi, indicative of non induction by E1A, or alternatively in an abundance below the sensitivity of the Northern technique. After restriction analysis, it was found that the lack of hybridisation to the RNA populations of some of the "uninduced" clones was due to the absence of detectable cDNA insert.

Eight cDNA clones were identified by sequence analysis. Three poorly induced clones that were partially sequenced were clone 2, 330 bp insert, clone 2, 270bp insert, clone 11, 427bp insert and clone 12, 160bp insert. Both inserts of clone 2 showed no similarity to any sequences within the EMBL or GENBANK databases and clones 11 and 12 were remarkably similar to a portion of CBP6, a yeast nuclear gene necessary for the synthesis of cytochrome b (Dieckmann and Tzagoloff 1985). The conserved region between clone 11 and CBP6 is within the coding region of CBP6. When translated, this region revealed 18 hydrophilic amino acids possibly in an α-helix configuration. Prediction of secondary structure is most successful when the putative protein is homologous with a protein of known structure. When only sequence is known, in the case of clone 11, without other information, such as function or the location of disulfide bonds, the accuracy of the prediction may be no better than 56% (Nishikawa 1983, Kabsch and Sander 1983).

Four strongly induced clones were partially sequenced. These clones, (clone 1, 530bp insert, clone 3, 330bp insert, clone 4, 239bp insert and clone 8, 170bp insert) were significantly similar to the structural coat protein genes of adeno-associated virus type 2 (AAV2). AAV2 is a defective parvovirus, which requires the presence of a helper virus, eg. adenovirus or SV40, for effective replication and will be discussed in detail

in chapter 4. Clone 1 and clone 3 each appear to be composed of 2 separate inserts. Apart from sequences which have similarity to AAV, each clone has sequences of 50 to 80 nucleotides which do not show significant similarity to any known gene. Evidence, obtained through Southern analysis, as presented below, suggests that the subsequences are cellular in origin. The presence of the unrelated inserts within the same clone may be a technical artifact. Clone 1 contains a stretch of three guanosines at position 51 in the left flanking sequence to the AAV sequence, at the junction of AAV and the "cellular" sequence. This is may indicate a cloning artifact from the initial dCTP tailing of cDNA before ligation to plasmid when constructing the library. There were no enzymatic manipulations of the dC-tailed inserts to encourage high fidelity annealing and ligation to dG-tailed plasmid. The annealing mixture was incubated at high temperature and allowed to cool slowly to produce annealed insert and plasmid. It is feasible that inserts with small overhangs of guanine residues would anneal to inserts with dC-tails instead of dG-tailed plasmids.

The alternative hypothesis is that the clones represent true junctions between integrated AAV and the cellular genome. Perhaps the integration event occured in a position within an actively transcribed cellular gene, such that the AAV sequences were transcribed as a part of the cellular gene. Perhaps a consequence of the integration event was the loss of transcription stop signals within the AAV sequences, resulting in transcription of adjacent cellular sequences. This would ultimately result in the presence of chimeric cDNAs in the cDNA library. Kotin and Berns (1989) isolated and sequenced genomic clones that represented AAV sequences integrated into the cellular genome during latency. The cellular DNA proximal to the viral DNA contained long polypurine tracts, believed to be a substrate for a cellular endonuclease activity, able to cleave a plasmid containing an AAV insert at the junctions with the vector *in vitro* (Gottlieb and Muzyczka 1988). Clone 1 and clone 3 both contain an extensive polypurine stretch adjacent to viral sequences.

Gene structure of clone 3, clone 5, and clone 11, was investigated by Southern analysis. Clone 3 strongly hybridised to a smear of DNA isolated from cells infected with AAV and WT, confirming the presence of AAV sequences within clone 3, since the pattern of hybridisation is typical of AAV DNA integration and replication in the cellular genome (chapter 4). Clone 3 also hybridised to specific sequences within uninfected and WT infected cellular DNA. This suggests the presence of cellular subsequences within clone 3, as discussed above. It is also possible that AAV sequences integrated into the cellular genome as a provirus until adenovirus infection, which would explain the specific hybridisation to uninfected cellular DNA. However, when the cells were infected by adenovirus, the alleged AAV provirus did not replicate and result in the typical pattern of hybridisation as shown with AAV and WT infected cellular DNA, implying that cellular sequences within clone 3 hybridised to specific cellular sequences within uninfected DNA. Thus, clone 3 is comprised of two unrelated sequences, an AAV sequence and an unidentified, probably cellular sequence.

Clone 5 also hybridised to specific sequences in uninfected and WT-infected cellular DNA. Clone 5 hybridised to AAV and WT infected cellular DNA in the typical pattern of AAV hybridisation, as mentioned above. Cross-hybridisation studies showed that clone 5 cross-hybridised to clone 4 (fig 3.2). Clone 4 was identified as containing AAV sequences, thus it is likely that clone 5 contains a similar region of AAV sequences.

Clone 11 hybridised to a specific genomic band of 3.8kb in DNA from uninfected, WT+AAV2 infected and WT infected cells. This implies that clone 11 contains a cellular sequence, even though the sequence was significantly similar to a yeast nuclear protein. A mammalian equivalent of this yeast nuclear protein has not been identified.

As the clone 11 sequence is similar to a small distinct region of the yeast gene, the homology may reflect a conserved structural or regulatory domain, rather that a possible contamination of yeast in the cells used to make the library. The predicted secondary structure of the translated region of conservation is an α-helix. From current literature, it is not known whether this region is an important regulatory or structural domain.

Summary

A subtracted cDNA library of 22,000 members was constructed to enrich for clones representing mRNA induced by E1A 40hrs after adenovirus infection. After differentially screening half of the library, a total of 244 clones were selected that appeared to represent cellular genes induced by E1A. 18 of 244 clones were chosen for further study. One third of these clones (6 of 18 clones) hybridised strongly to specific RNA species isolated from WT infected cells and not dl 312 (E1A negative) infected cells, implying that the clones were induced by E1A. The level of induction of the other 12 clones was undetectable by Northern analysis. Sequencing of induced clones, and Southern analysis revealed the presence of AAV 2 sequences in a number of clones. This suggests that the original virus stocks, or cells used to make the subtracted library, were contaminated by AAV2. The significance of this is discussed in greater detail in chapter 4.

Chapter 4

AAV interaction with adenovirus during productive infection

Introduction

As mentioned in chapter 3, four clones, isolated from a subtractive library, enriched for E1A-induced cellular sequences, showed remarkable sequence similarity to adeno-associated virus type 2 (AAV2). Adeno-associated viruses were believed to be degradation products or precursors of the adenovirus virion when first identified in the late 1950s, but by the mid-1960's AAV was recognised as a distinct virus, dependent on adenovirus coinfection for productive infection (Atchison *et al.* 1965, Mayor *et al.* 1965). The adeno-associated viruses are members of the family Parvoviridae, in the genus dependoviruses, aptly named for their complete dependence on coinfection with a helper virus (adenovirus, vaccinia, herpes simplex virus or human cytomegalovirus) for productive infection (Atchison *et al.* 1965, Rose and Koczot 1972, Schlehofer *et al.* 1986, Buller *et al.* 1981, McPherson *et al.* 1985).

AAV are amongst the smallest DNA animal viruses. The single-stranded linear DNA genome is encapsidated in an icosahedral structure, approximately 20nm in diameter (Mayor *et al.* 1965). The viral genome contains 4681 bases. There are two open reading frames (ORFs). The ORF that extends over the left half of the genome codes for replication proteins. The right ORF codes for structural proteins. At the end of the genome there are inverted terminal repeats of 145bps (Koczot *et al.* 1973, Lusby *et al.* 1980). The terminal 125bps are palindromic, but the overall palindrome is interrupted by two shorter palindromes in the centre of the larger palindrome, forming a T-shaped structure after maximum base pairing. This structure is used as a primer for DNA replication (Hauswirth and Berns, 1977). In the absence of helper virus, the AAV genome integrates efficiently into the cellular genome and can exist as a provirus for many cellular generations until rescue with a helper virus (Hoggan *et al.* 1972, Berns *et al.* 1975, Handa *et al.* 1977).

The relationship between AAV, the helper virus and the cell is dynamic. While AAV replication is reliant on the presence of the helper virus, replication and transcription of the helper virus are affected by AAV replication. When AAV is coinfected with adenovirus , the production of adenovirus virions is reduced, due to the inhibition of adenovirus DNA replication (Carter *et al.* 1979). AAV is also responsible for the reduction of cell multiplication in the absence of helper virus. Winocour *et al.* (1988) showed that AAV infection of presenescent embryonic fibroblasts resulted in an accumulation of cells in the late S or G2 phase of the cell cycle and a significant inhibition of cell division. Although AAV significantly affects cell function in the absence of adenovirus infection, and adenovirus kinetics during coinfection, it is often undetected due to the lack of common assay methods, such as plaque assays.

In this chapter evidence is presented that AAV had infiltrated a large number of adenovirus stocks possibly through the cell lines used to propagate the virus stocks. Coinfection of rat embryo fibroblasts with WT adenovirus contaminated with AAV, confirmed reports in the literature that replicative intermediates of AAV are formed during productive infection, concomitant with possible integration into the cellular genome. RNA transcription was also investigated during a productive AAV infection. The five clones mentioned above mapped to 75-90 mu of the genome and recognised all AAV transcripts. The kinetics of AAV transcription during infection was investigated through Northern analysis of RNA from cells isolated at different times after infection. In order to isolate cellular genes induced by adenovirus, a portion of the subtractive library was screened for AAV sequences and differentially screened with cDNA from uninfected, dl 312 infected or WT (without AAV) infected cells.

AAV contamination within adenovirus stocks

AAV are amongst the smallest DNA animal viruses. The AAV virion is an icosahedral structure, approximately 20nm in diameter (Mayor *et al.* 1965). When the

WT-adenovirus stocks used for experiments were examined under electron microscopy, a large number of 20nm particles, which resemble AAV particles, were detected as shown in figure 4.1. To investigate the extent of AAV contamination in all of the available adenovirus stocks, DNA was isolated from virus infected HeLa cell lysates by treatment in alkali, applied to nylon membranes in a slot configuration and probed with AAV as described in 2.2.6. Twenty eight different adenovirus mutants were tested in this manner and eighteen viruses hybridised to AAV sequences (64%), as displayed in figure 4.2. Not all adenovirus mutants have the same growth kinetics as WT when infecting HeLa cells, so the filters were also probed with adenovirus, to confirm the presence of virus. The results show that AAV had infiltrated a significant proportion (64%) of adenovirus mutant stocks. AAV requires the presence of a functional E1 region to replicate (Laughlin *et al.* 1982). Mutant viruses lacking a functional E1 region were propagated on 293 cells which constitutively express adenovirus E1 proteins, thus supplying the requirements for AAV replication (Graham *et al.*, 1977) as demonstrated in table 4.1.

To identify the source of contamination, DNA from uninfected REFs and from cell lines used to propagate virus stocks, eg. 293 cells and different stocks of HeLa cells, were digested with *Hin*d III and *Sma*1, Southern transferred and probed with the AAV genome as described in 2.2.6. and 2.2.7. In the absence of helper virus during an AAV infection, AAV virions adsorb and penetrate the cell membrane and are transported to the nucleus, where they become uncoated (Rose and Koczot 1972). The AAV genome integrates very efficiently into different locations in the cellular genome in approximately 30% of the cell population, and can exist as a provirus for many cellular generations until rescue with helper virus (Hoggan *et al.* 1972, Berns *et al.* 1975). As indicated in figure 4.3, the AAV genome was not detected in primary rat embryo fibroblasts, but was detected in one of the HeLa cell lines. There was no evidence of AAV latent infection in 293 cells, however not all 293 stocks were comprehensively checked for the presence of AAV.

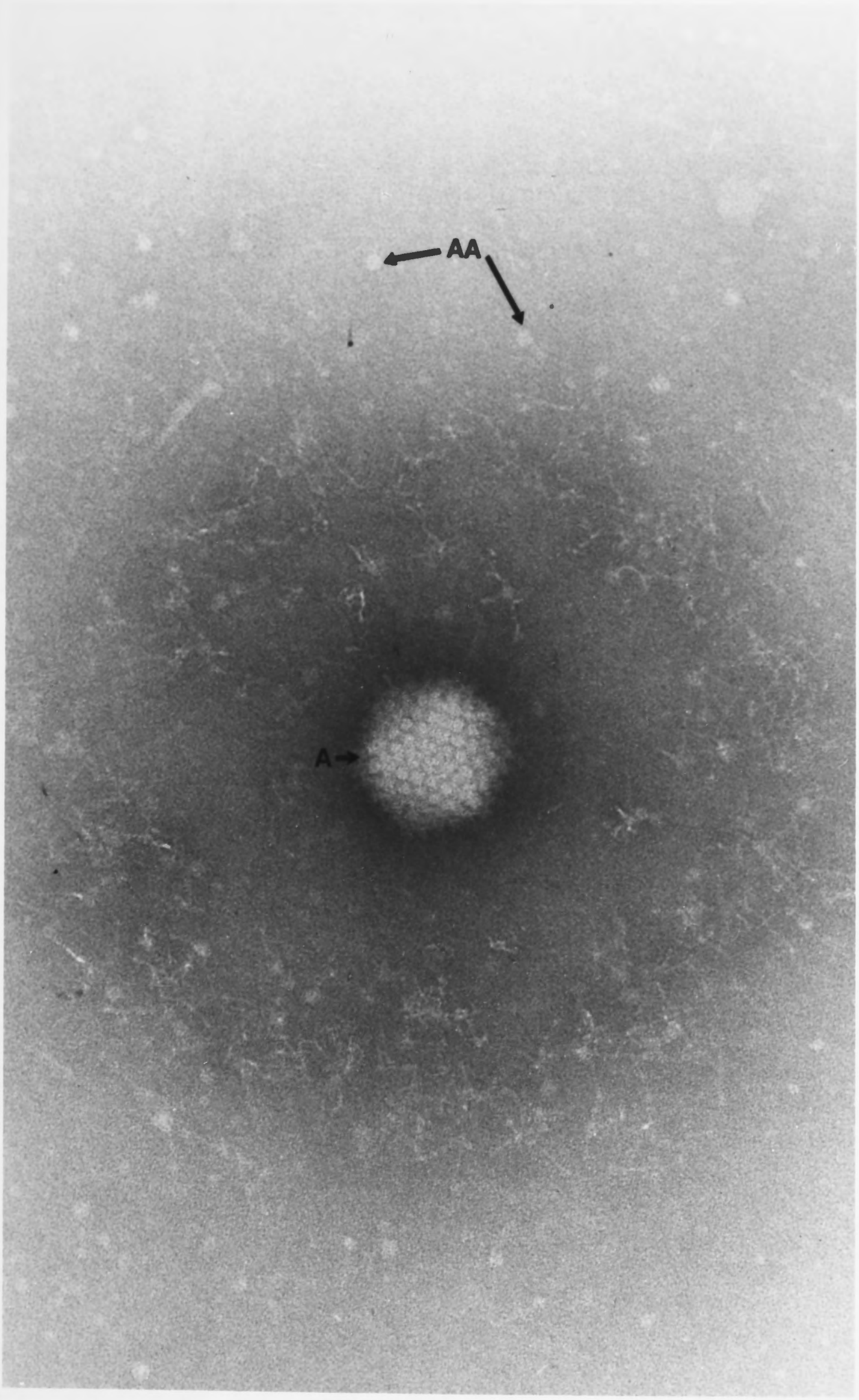


Figure 4.1. Electron micrograph of WT viral stock.
Virus particles were spread onto pure carbon-coated, glow-discharged, 400 mesh copper grids, and negatively stained. The micrograph was taken on a Philips 301 electron microscope. Total magnification x320,000
A- adenovirus
AA- adeno-associated virus

Figure 4.2 Extent of AAV contamination within virus stocks.
DNA from 28 different adenovirus mutants was isolated from virus infected cell lysates by treatment in alkali and applied to nylon membranes in a slot configuration. A. probed with the AAV genome. B. probed with the WT Ad 5 genome. C. probed with cellular genomic DNA. Eighteen viruses hybridised to AAV sequences (64%). This suggested that AAV had infiltrated a significant proportion of adenovirus mutant stocks.

Virus	A AAV	B WT	C Cell	Virus	A AAV	B WT	C Cell
WT				E1APM928			
dl 312				PM975			
dl 313				in 500			
dl 327				hr6			
dl 347				hr1			
dl 348				hr3			
dl 358				hrA			
dl 355				hr7			
12S				309 RH87			
13S				309 RH			
12SPM928				309 109			
12S 705				309 21			
12S 961				309 3			
ts 36				309 64			

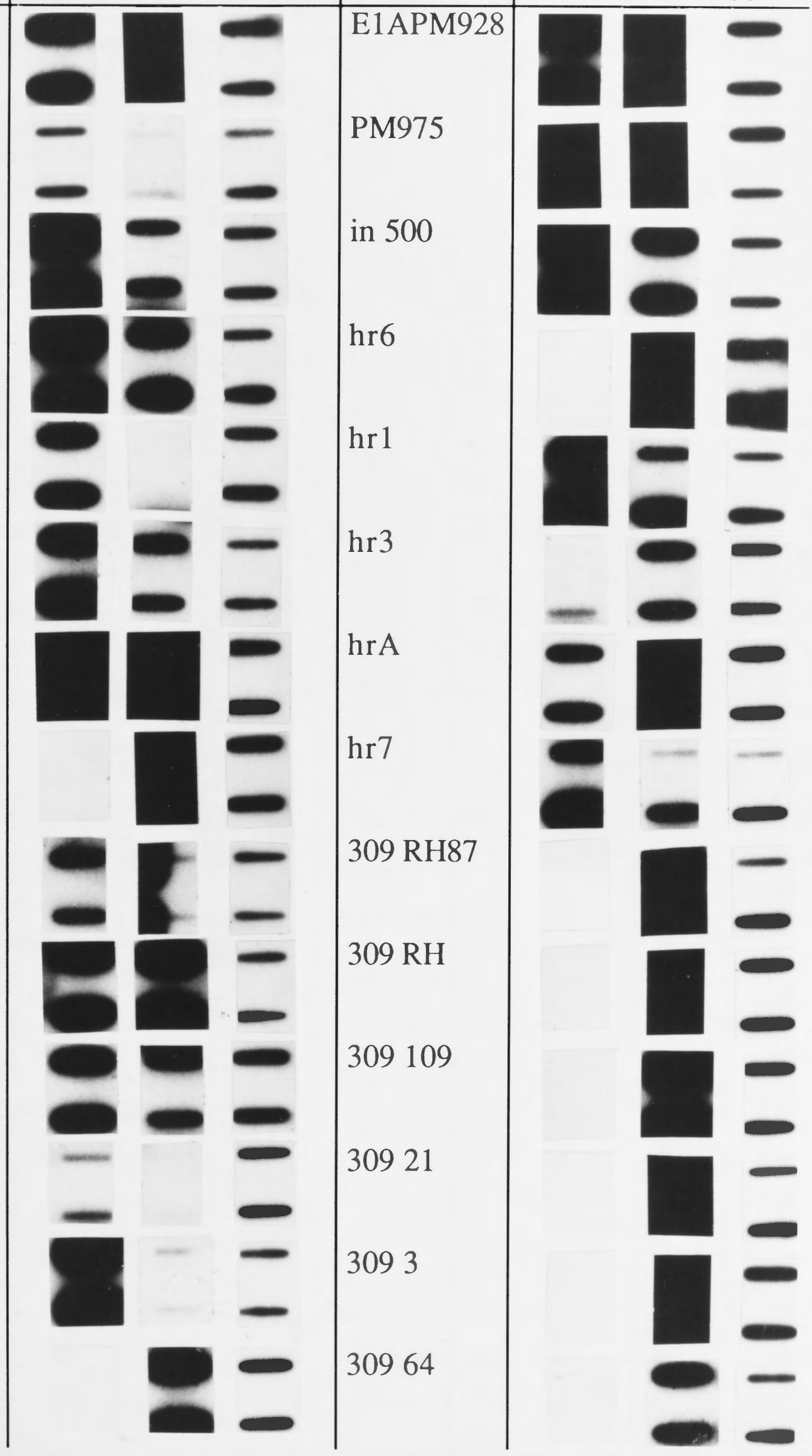


Table 4.1 AAV cross contamination

+ -AAV detected

- -AAV not detected

Virus	Mutation	Propagation	AAV presence
WT		HeLa	+++
E1A			
dl 312	E1A negative	293	+
dl 347	only 243aa E1A product	293	++
12S	only 243aa E1A product	293	+
12S PM928	aa 124 substitution in CD2 no 289 E1A product	293	++
12S 705		293	+
12S PM961	aa 135 substitution in 243 product. no 289 E1A	293	+++
in 500	truncated 289 aa,normal 243 aa	293	+++
hr1	truncated 289aa, normal 243aa	293	+++
E1APM928	aa 124 substitution in CD2 both E1A products	293	+++
dl 348	only 289aa E1A product	293	+++
13S	only 289aa E1A product	293	+++
PM975	only 289 aa E1A product	293	+++
hr3	aa substitution in CD3 in 289aa E1A	293	---
hrA	no 243aa, truncated 289aa E1A	293	++
E1B			
dl 313	E1B negative	293	++++
hr6	no 495aa E1B	293	---
hr7	no 495aa E1B, reduced level of other E1B proteins	293	++
E2			
ts 36	thermolabile DNA binding protein	HeLa	---
E3			
dl 327	E3 negative	HeLa	++++
E4			
dl 355	no 34kDa E4 product	HeLa	---
dl 358	deletion in ORF 1 of E4	HeLa	++++
309 RH87	Mutations in E1A enhancer regions	293	---
309 RH	" "	293	---
309 109	" "	293	---
309 21	" "	293	---
309 3	" "	293	---
309 64	" "	293	---

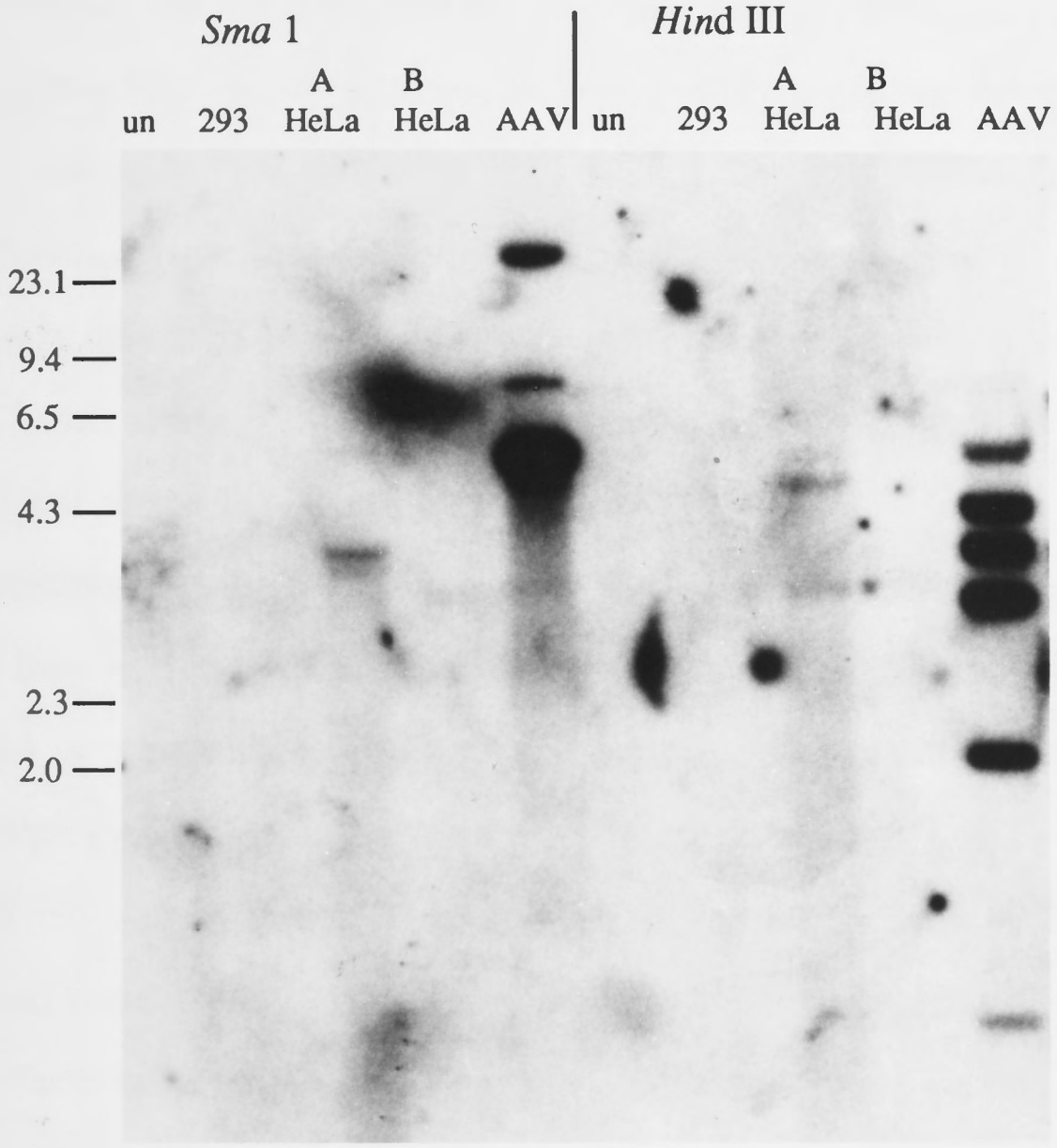


Figure 4.3. AAV sequences within cell lines .
20μg DNA from uninfected REFs (un) and from cell lines used to propagate virus stocks, eg. 293 cells and different stocks of HeLa cells (A and B), were digested with *Hin*d III and *Sma*1, Southern transferred and probed with the AAV genome. AAV genomic DNA was added to 10μg rat genomic DNA at a concentration of 1 copy per cell and digested with the appropriate enzymes in the AAV track.

AAV replication during adenovirus infection

The AAV genome is linear, single-stranded and contains 4681 bases. The organisation of the genome is shown in figure 4.4. There are two open reading frames (ORFs). The ORF that extends over the left half of the genome codes for replication proteins. The right ORF codes for structural proteins. At each end of the genome there are inverted terminal repeats of 145 bases (Koczot *et al.* 1973, Lusby *et al.* 1980). The terminal 125bps are palindromic, but the overall palindrome is interrupted by two shorter palindromes in the centre of the larger palindrome, forming a T-shaped structure after maximum base pairing. This structure is used as a primer for DNA replication (Hauswirth and Berns, 1977).

AAV replication during productive infection has been described in immortalized human cell lines or chinese hamster cell lines. The parental single-stranded (SS) DNA is converted to a parental double-stranded replicative form (RF), from which is synthesised progeny RF monomers, dimers and concatemers (Laughlin *et al.* 1982, Straus *et al.* 1976), by a "rolling hairpin" model of DNA replication, as described by Tattersall and Ward (1976). The progeny plus and minus DNA strands are displaced from the RF and are separately packaged within preformed empty capsids, which then mature to stable viral particles (Myers and Carter 1980). Experiments were performed to characterise AAV replicative forms at 40hrs of productive infection in primary REFs.

In order to identify AAV replicative forms, Southern analysis was performed on DNA from cells infected with the contaminated WT stock (WT+AAV), and uninfected cells. Genomic DNA was isolated from uninfected cells, cells infected with WT and infected with WT+AAV, 40hrs pi. The DNA was digested with *Sma*1, electrophoresed through agarose, transferred to nylon membranes and probed with the AAV genome, as described in section 2.2.6 and 2.2.7. *Sma*1 restriction sites are in the inverted terminal repeats (ITR) of the AAV genome, and liberate the monomer genome after

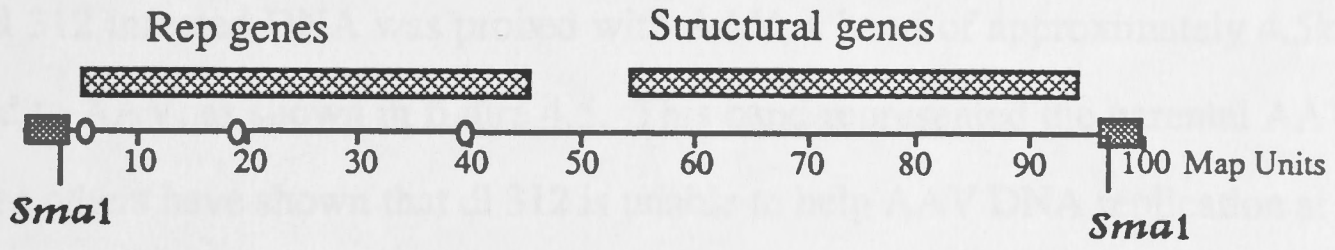


Figure 4.4 Genomic organisation of AAV.
The AAV genome is linear, single-stranded and contains 4681 bases. There are two open reading frames (ORFs). The ORF that extends over the left half of the genome codes for replication proteins. The right ORF codes for structural proteins. There are 3 promoters, mapped to 5, 19 and 40 map units (mu). At each end of the genome there are inverted terminal repeats of 145 bases.

▩ -Inverted terminal repeat ▩ -Open Reading Frame
0 -Promoter *Sma*1 -*Sma*1 restriction enzyme sites

digestion. As both polarities of viral genomic DNA are packaged equally into separate genomes, during extraction the complementary strands can base pair to form duplex DNA, thus becoming sensitive to restriction enzyme analysis. Alternatively, Marcus-Sekura and Carter (1983) detected duplex AAV DNA in the absence of helper virus and in the presence of DNA synthesis inhibitors, believed to represent annealed parental single strands, or integration of the AAV single strands into cellular chromosomes, resulting in restriction enzyme sensitivity.

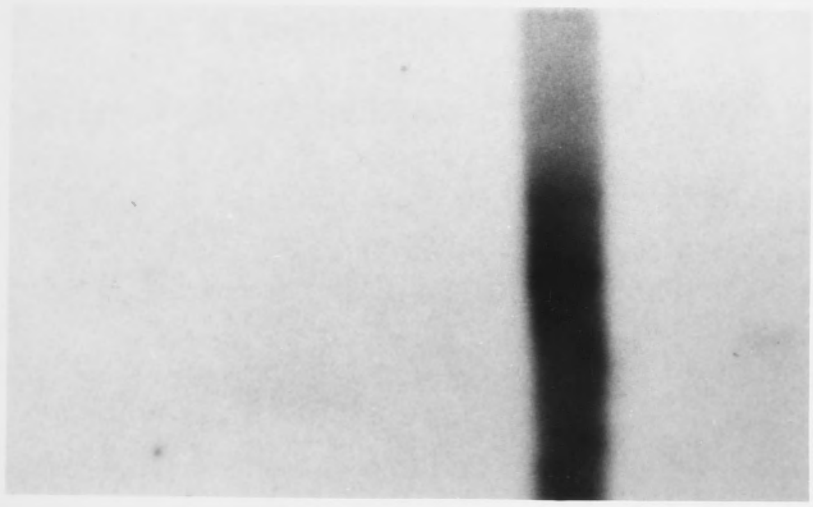
When dl 312 infected DNA was probed with AAV, a band of approximately 4.5kb hybridised to AAV, as shown in figure 4.5. This band represented the parental AAV genome, as others have shown that dl 312 is unable to help AAV DNA replication at a low MOI (Laughlin *et al.* 1982). Laughlin and her colleagues (1982) found that most of the infecting AAV genomes were degraded, 20hrs after coinfection with dl 312 (MOI 10) in human KB cells. There was no evidence of degradation of the AAV genome after 40 hrs of co-infection of dl 312 (MOI 25) in 3°REFS, as shown in figure 4.5. AAV was present in the original dl 312 viral stock because the 293 cells used to propagate dl 312 virus, supplied the E1A functions necessary for AAV replication during propagation.

Figure 4.5 also shows a general smear of hybridisation unique to DNA from cells infected with WT and AAV. This smear has been detected by other investigators during AAV and WT coinfection of 293 cells and KB cells (Redemann *et al* 1989). Hybridisation to DNA of lower molecular weight than the monomer AAV was not unexpected, as variant DNA molecules, generated during replication, ranging from 3% to 100% of the genome length have been identified and characterised (Hauswirth and Berns 1979, de la Maza and Carter 1980a). Hybridisation of AAV to a smear of DNA of higher molecular weight than the monomer genome may be the result of a number of different interactions. The rolling hairpin model of replication allows for concatemeric generation of SS progeny genomes by strand displacement. Conceivably, a variable number of AAV DNA molecules of higher molecular weight than the AAV monomer,

A.

Sma I

un | WT -AAV | WT +AAV | dl



>23kb

6.8kb

B.

Sma I

un | WT -AAV | WT +AAV | dl

4.5kb

Figure 4.5 Southern analysis of infected REFs 40 hrs after infection. 10μg DNA, from uninfected cells, cells infected with WT and infected with WT and AAV, 40hrs pi. was digested with *Sma*1, Southern transferred to nylon membranes and probed with the AAV genome. *Sma*1 restriction sites are at the termini of the AAV genome, and liberate the monomer genome after digestion. When dl 312 infected DNA was probed with AAV, a band of approximately 4.5kb hybridised to AAV. There is a general smear of hybridisation unique to DNA from cells infected with WT and AAV.
A.Underexposure revealing two bands of >23kb and 6.8 kb in the WT + AAV track.
B. Overexposure to detect 4.5kb band in dl 312 track

may be isolated after *Sma*1 digestion, dependent on whether both ITRs of a progeny strand have been replicated at the time of isolation. Perhaps recombination events have occurred between parental/progeny viral genomes, resulting in the generation of variable sized genomes. Alternatively, AAV DNA may have integrated into the cellular genome, losing one of the ITRs during the imperfect integration, resulting in an apparent increase in genome size.

An underexposure of the smear revealed a band of size 6.8kb and no obvious band equal to the size of the monomer genome, dimers or concatemers. To establish whether the 6.8kb band represented a replicative intermediate or a variant AAV with an unusually large genome, resulting from recombination with cellular DNA or virus, DNA from viral stocks were isolated and examined. Viral stocks (infected cell lysates) were digested with micrococcus nuclease. Encapsidated viral DNA is completely resistant to micrococcal nuclease (de la Maza and Carter 1980b). After phenol extraction, to release viral DNA, electrophoresis and transfer to nylon membranes, the viral DNAs were probed with AAV. All viral stocks contained AAV of the expected size of 4.7kb after nuclease digestion as shown in figure 4.6. The viral DNA from lysates that were not nuclease digested, contained the monomer genome and AAV DNA of higher molecular weight that was not present in the nuclease treated sample and therefore not encapsidated. Although there was not a large detectable population of 6.8kb AAV DNA in the untreated samples, the result suggests that the 6.8kb band from Southern analysis may represent a defective variant DNA molecule that does not become packaged into virions or a replication intermediate. The cells were infected at the same time with WT+AAV, thus AAV DNA replication was likely to be synchronous at 40hrs pi. A significant proportion of AAV DNA may be at the same stage of strand displacement, yielding a 6.8kb fragment.

Hybridisation of AAV to a smear of DNA of higher molecular weight than the monomer genome (figure 4.5) may be the result of concomitant imperfect random integration of a proportion of AAV genomes into the cellular genome. The lack of

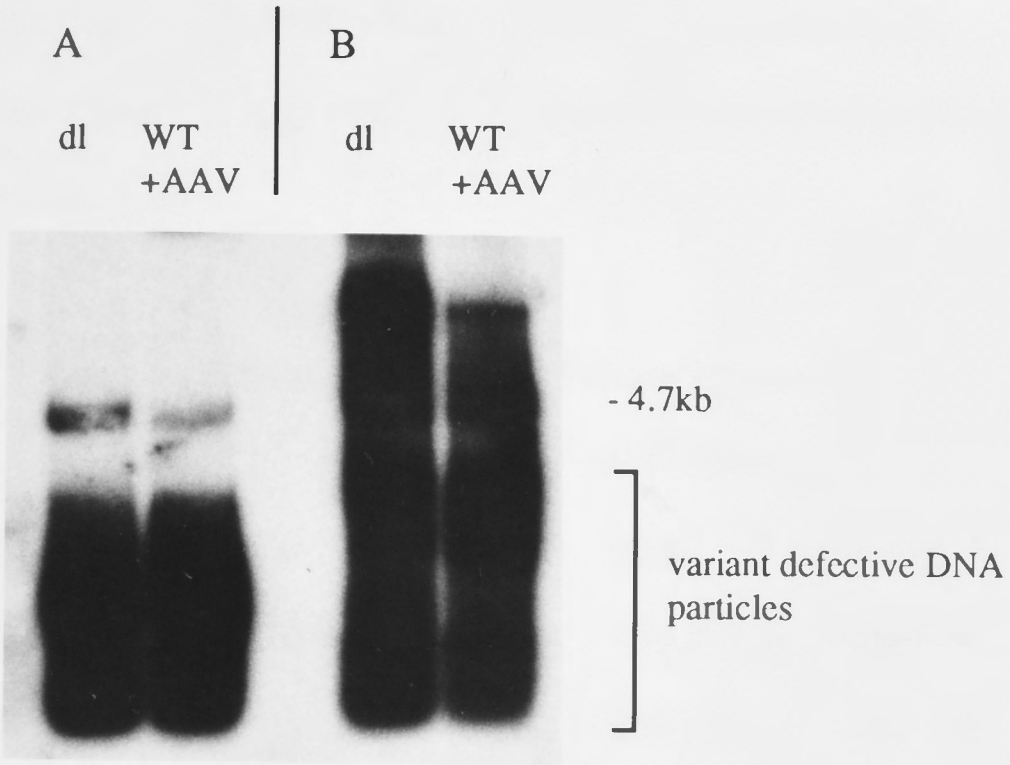


Figure 4.6 DNA from infected cell lysates
DNA from viral stocks were examined for the presence of high molecular weight AAV genomes. A. DNA from dl 312 infected cell lysate (dl) and DNA from WT+AAV infected cell lysates were digested with micrococcal nuclease, resulting in degradation of unencapisidated DNA . B. dl and WT+AAV DNA from infected lysates not digested with micrococcal nuclease. The membrane was probed with the AAV genome

concatemeric structures and hybridisation to high molecular DNA (>23kb) may be the result of integration involving the loss of a *Sma*1 site and replication of adjacent cellular sequences. The smearing of high molecular weight DNA may also be the result of homologous reciprocal recombination. The replication of AAV may promote reciprocal crossovers between episomal AAV and/or integrated forms, leading to expanded or contracted AAV genomes. Recombination through short homologous sequences or non homologous recombination, may result in AAV genomes of variable length during productive infection.

The expression of AAV gene products during a productive infection

During productive infection, 3 major RNA species of 2.3, 3.3 and 3.9kb are transcribed from the AAV genome, also found in unspliced forms of 2.6, 3.6 and 4.2kb respectively (Marcus *et al.* 1981). These RNA species are transcribed from 3 promoters, mapped to 5, 19 and 40 map units (mu) (Green and Roeder 1980, Green *et al.* 1980, Laughlin *et al.* 1979), as shown in figure 4.7. RNAs encoding for structural coat proteins (2.6kb and 2.3kb) are transcribed from the promoter at 40 mu, while transcripts from the promoters at 5 and 19 mu are involved in DNA replication and regulation. The RNAs share a common intron between 41 to 48 mu and a common polyadenylation site at 95 mu. As mentioned in chapter 3, at least 4 clones isolated from a subtractive library, enriched for sequences induced by E1A, showed significant similarity to AAV. All of the sequenced clones mapped between 75.8 and 91 mu as shown in figure 4.7, and were able to detect all RNA species when used as probes in Northerns. In order to investigate transcription from the three promoters during productive infection, total cytoplasmic RNA was isolated from dl 312 and WT+AAV infected cells at various times after infection, Northern transferred and probed with the clones, shown to be similar to AAV, as described in section 2.2.4.and 2.2.7.

The clones were broadly divided into 2 groups on the basis of the hybridisation pattern obtained. Clones 1, 3 and 9 showed the same hybridisation pattern as shown in

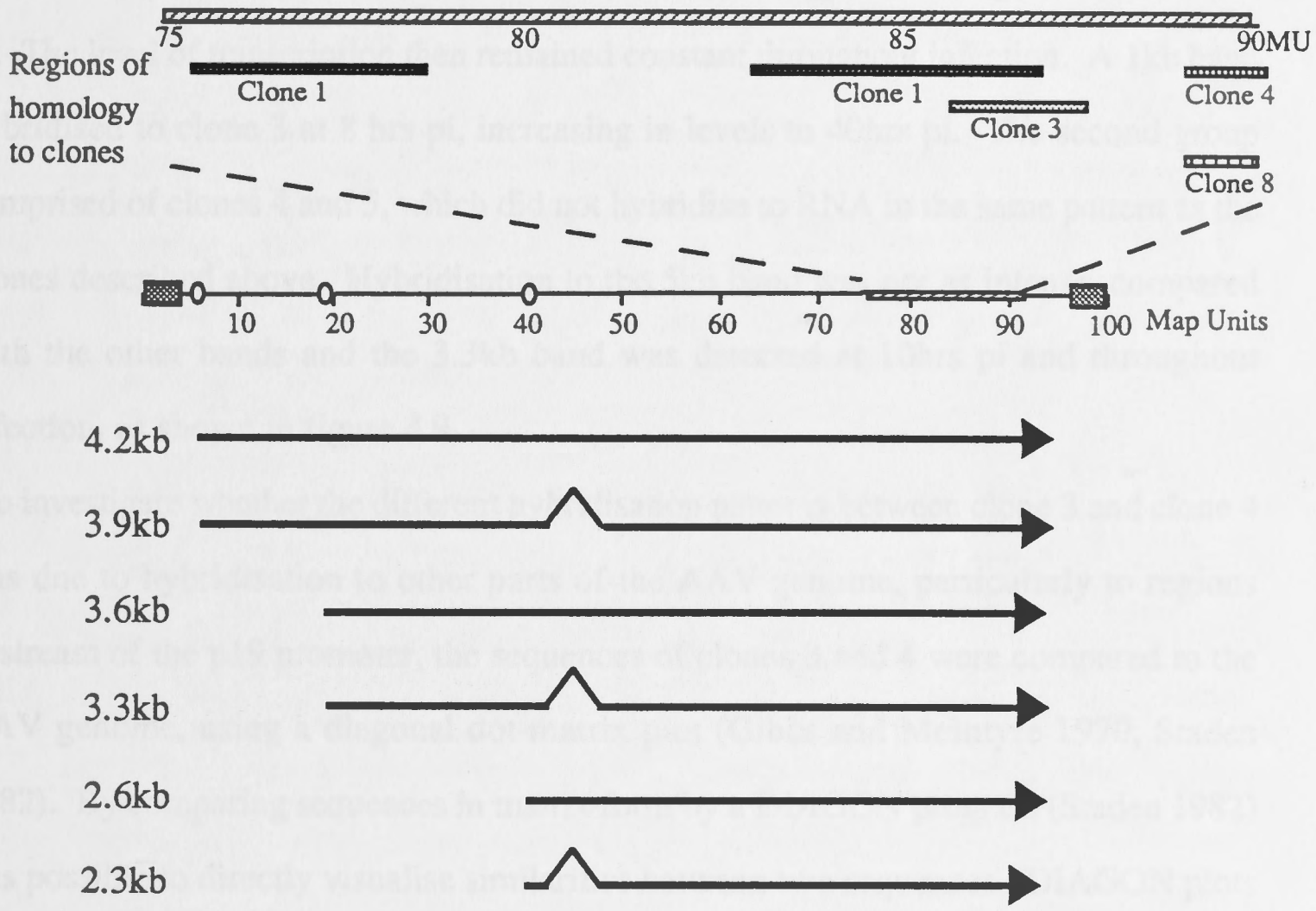


Figure 4.7. Transcription map of AAV 2
3 major RNA species of 2.3, 3.3 and 3.9kb are transcribed from the AAV genome, also found in unspliced forms of 2.6, 3.6 and 4.2kb respectively. RNAs encoding for structural coat proteins (2.6kb and 2.3kb) are transcribed from the promoter at 40 mu, while transcripts from the promoters at 5 and 19 mu are involved in DNA replication and regulation. The RNA share a common intron between 41 to 48 mu and a common polyadenylation site at 95 mu.

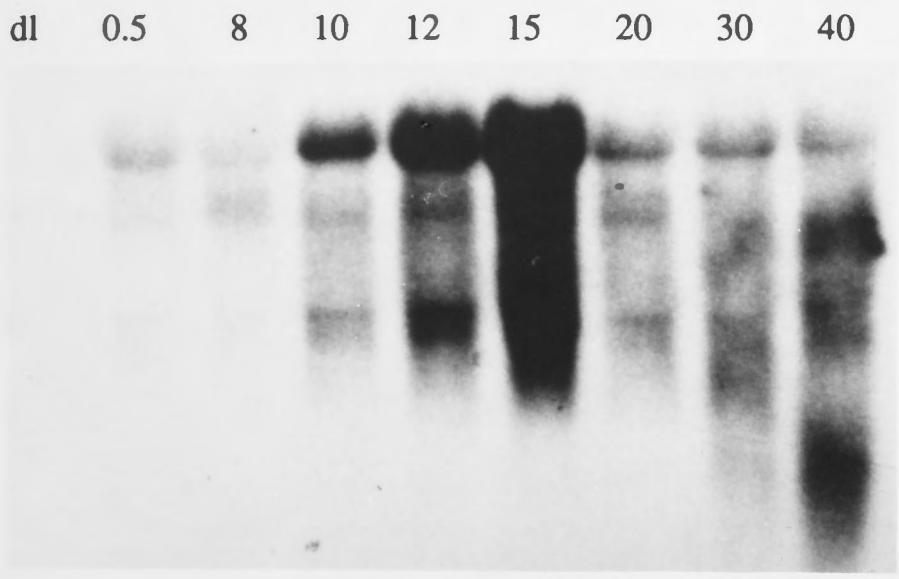
^ - Splice site ▩ - Inverted terminal repeats
0 - Promoter ▭ - Region of homology to AAV clones

figure 4.8. A transcript of approximately 5kb appeared within the first 30mins after coinfection with WT. The level of transcription increased at 10 hrs pi and remained constant through the rest of the infection. The 4.2 and 3.9kb transcripts were first detected at 8hrs pi at a comparably low level which remained constant through infection. At 30-40hrs pi a novel band was clearly detected with clones 3 and 9. This band represented a transcript of approximately 3.3kb. The transcripts encoding for structural proteins (2.6 and 2.3kb) were detected at 8hrs pi, increasing in level at 10hrs pi. The level of transcription then remained constant throughout infection. A 1kb band hybridised to clone 3 at 8 hrs pi, increasing in levels to 40hrs pi. The second group comprised of clones 4 and 5, which did not hybridise to RNA in the same pattern as the clones described above. Hybridisation to the 5kb band was not as intense, compared with the other bands and the 3.3kb band was detected at 10hrs pi and throughout infection, as shown in figure 4.9.

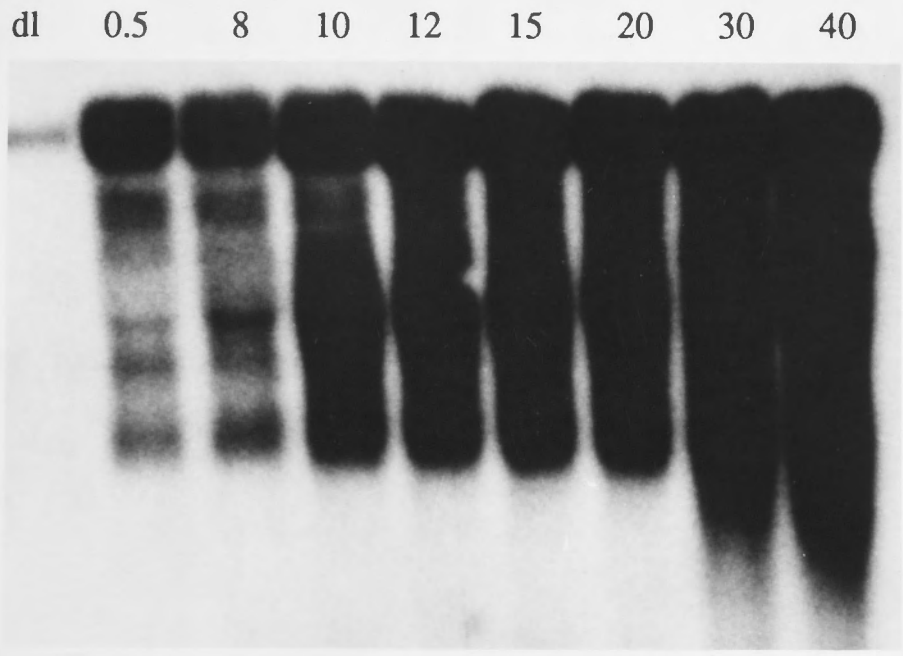
To investigate whether the different hybridisation patterns between clone 3 and clone 4 was due to hybridisation to other parts of the AAV genome, particularly to regions upstream of the p19 promoter, the sequences of clones 3 and 4 were compared to the AAV genome, using a diagonal dot-matrix plot (Gibbs and McIntyre 1970, Staden 1982). By comparing sequences in matrix form by a DIAGON program (Staden 1982) it is possible to directly visualise similarities between two sequences. DIAGON plots of clone 3 and clone 4 compared to the AAV genome were employed Sequence matches of 15 nucleotides, sufficient for hybridisation, were searched for, and as shown in figure 4.10, only known homologies were detected.

These results suggest that AAV transcription from the promoter at 5mu occurred shortly after coinfection with adenovirus. Transcription of RNA from the promoter at 40mu was detected from 8-10hrs pi, while transcription from the promoter at 19mu increased at 30-40hrs from a constitutive level detectable at 10hrs. A novel transcript of 1kb was also detected from 10hrs.

A. probed with clone 1



B. probed with clone 3



C. probed with clone 9

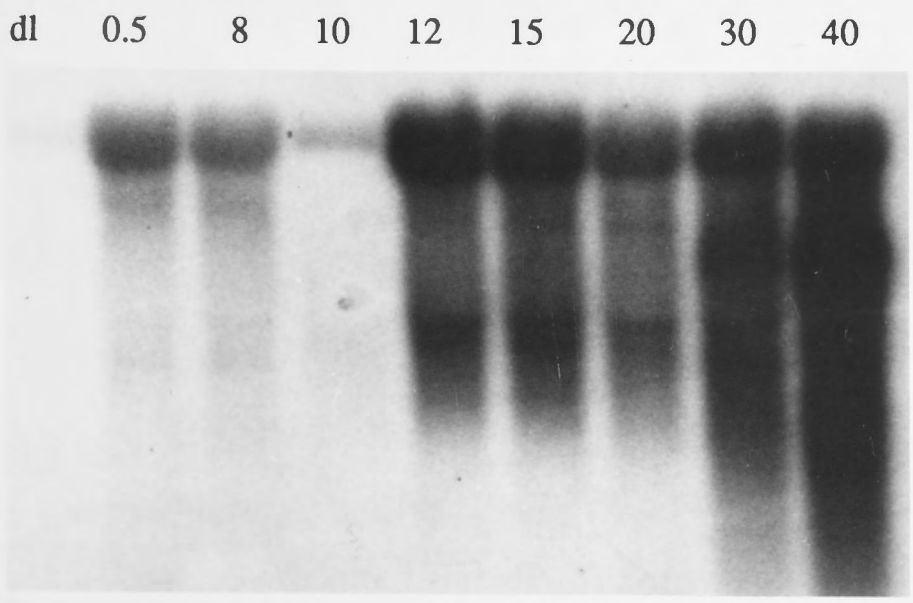
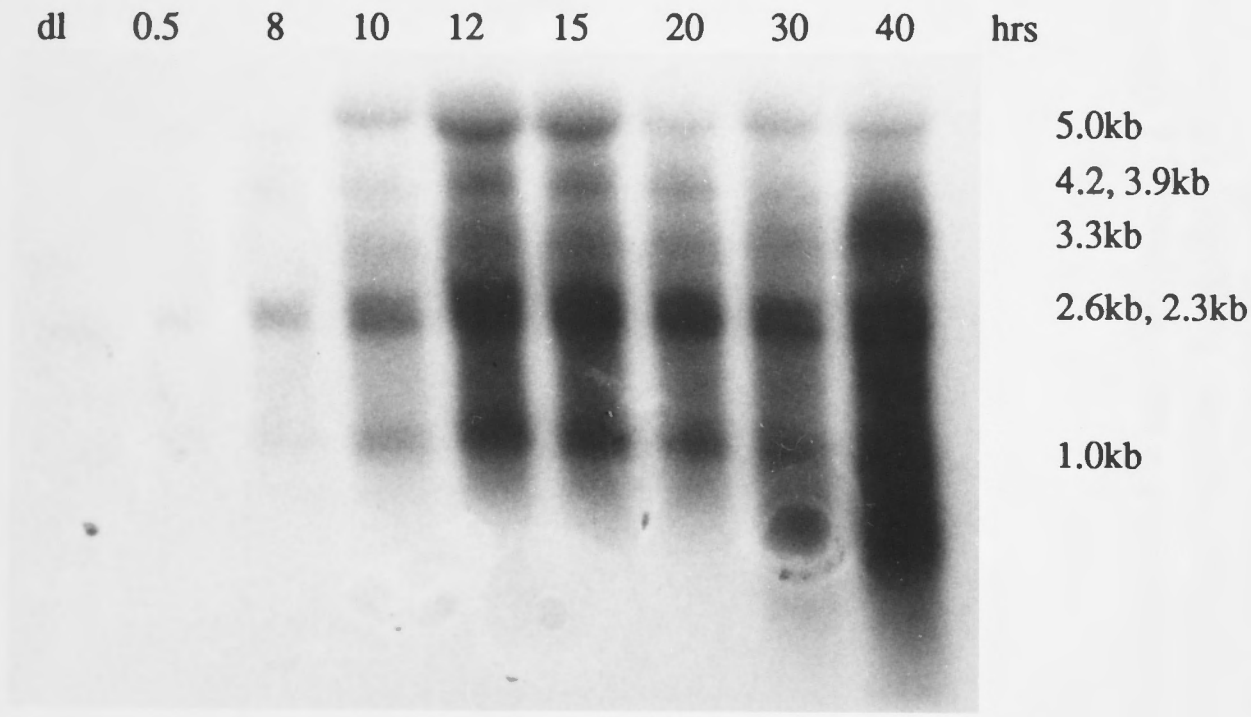


Figure 4.8. Expression of AAV gene products during co-infection with WT. 20μg of total cytoplasmic RNA, from dl 312 infected cells 40hrs pi (dl) and from WT+AAV infected cells at various times after infection, were Northern transferred and probed with A. clone 1, B. clone 3, and C. clone 9
Size of transcripts are indicated.

A. probed with clone 4



B. probed with clone 5

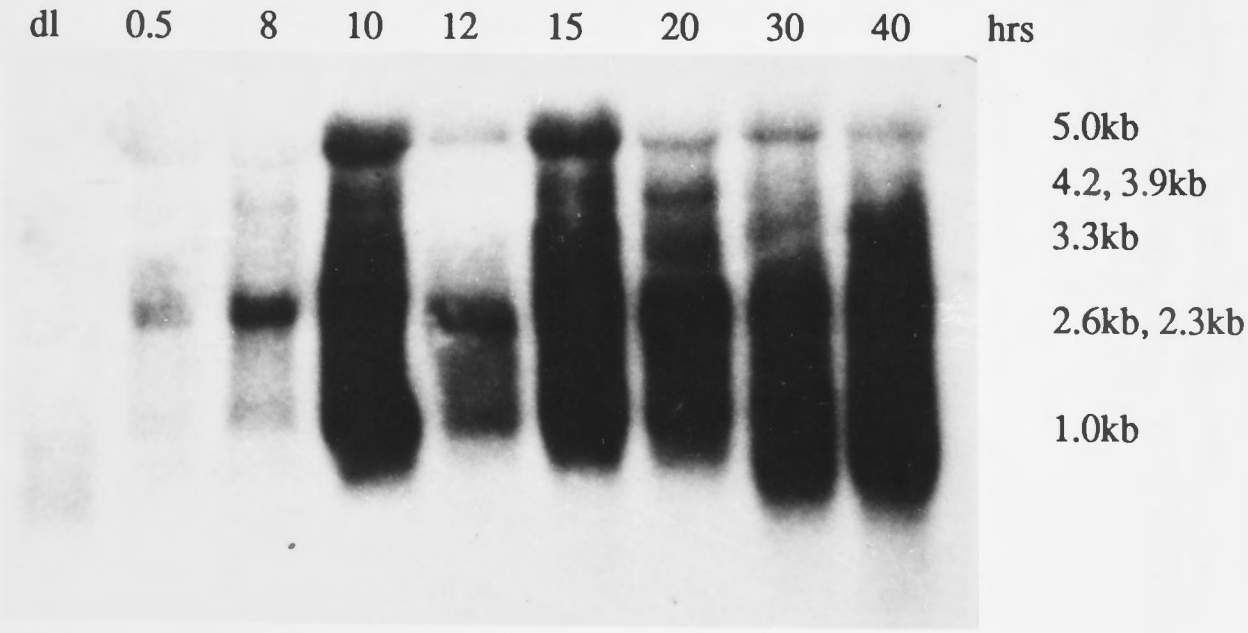


Figure 4.9 Expression of AAV gene products during co-infection with WT. 20μg of total cytoplasmic RNA, from dl 312 infected cells 40hrs pi (dl) and from WT+AAV infected cells at various times after infection, were Northern transferred and probed with A. AAV clone 4 and B. clone 5 Sizes of transcripts are indicated.

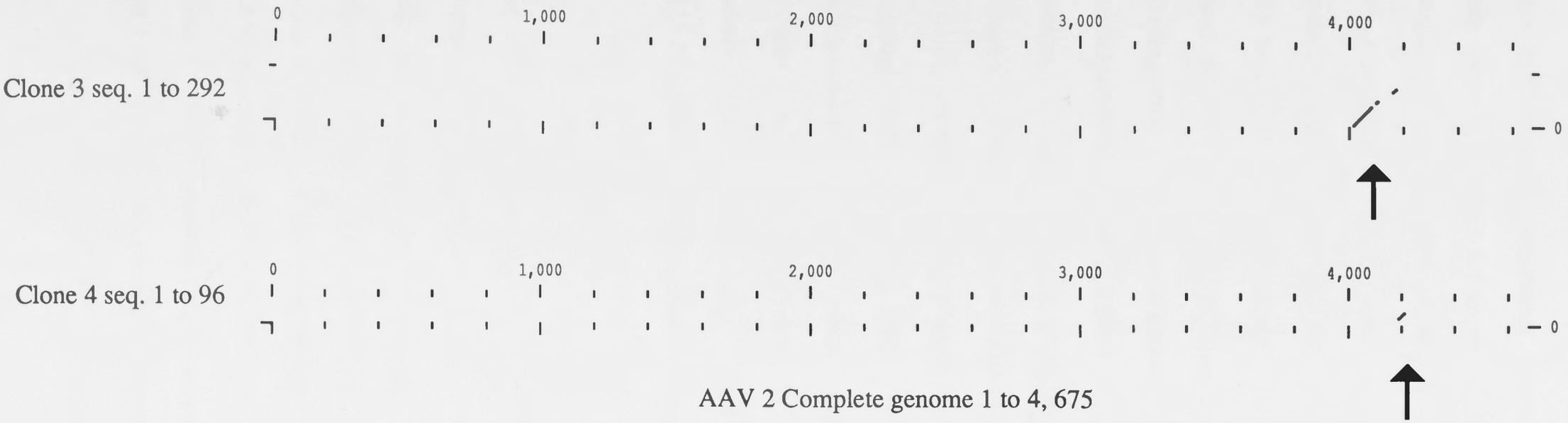


Figure 4.10 Comparison of the sequences of clones 3 and 4 to AAV sequences using a DIAGON plot. The stringency was set at 15 nucleotides in a window of 15 nucleotides. Both clones do not show homology to other sections of the AAV genome apart from homologies at 75-90mu, as shown by →

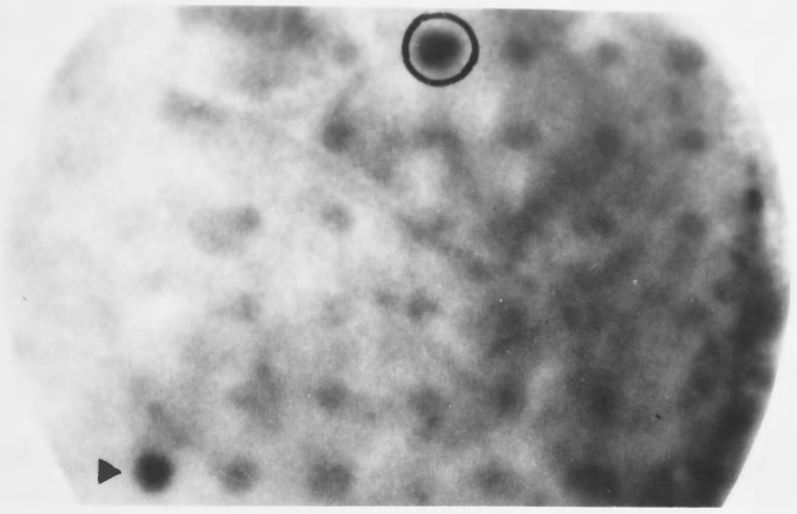
Extent of AAV contamination among differentially expressed clones

Northern analysis and sequencing of E1A-induced clones in chapter 3 has revealed that six of eighteen clones were similar to AAV sequences. To investigate the extent of AAV sequences within the remaining differentially screened clones, a proportion of clones, isolated during the initial differential screenings, were rescreened with AAV. 77 clones, which were isolated from three rounds of screening with E1A positive and E1A negative cDNA probes, as described in chapter 3, were grown on nitrocellulose filters and probed with the AAV genome. This screening strategy was repeated three times to identify 55 clones from 77, that shared strong sequence homology with AAV. 19 clones were consistently negative through the 3 rounds of screening. In order to isolate cellular sequences influenced by adenovirus infection in the presence or absence of AAV, the 19 non-AAV sequences were probed with radiolabelled cDNA made from RNA isolated from uninfected cells and cells infected with WT, dl 312 and WT+AAV as described in section 2.2.5. One clone (pTD20) from 19 clones hybridised specifically to cDNA from uninfected, dl 312 infected and WT infected cells, implying that one clone is cellular in origin and in sufficient abundance to be detected by the cDNA probes. However there was no evidence of differential induction of pTD20, as shown in figure 4.11. Nonetheless, this clone was analysed in depth in chapter 5.

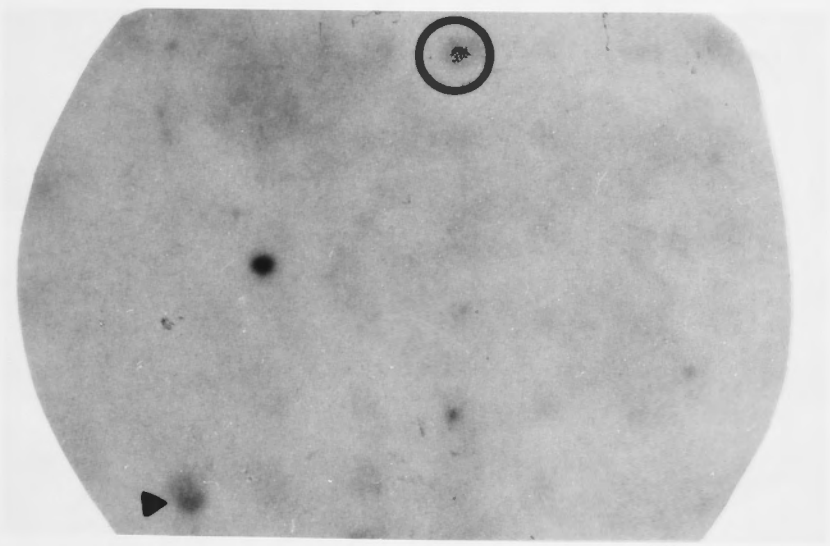
Discussion

AAV contamination was initially detected by sequencing E1A induced clones, isolated by differential screening of a subtracted cDNA library. Investigation of different viral stocks for AAV sequences suggested that AAV infiltration was extensive, and the possible source of the contamination was traced to a HeLa cell line, used to propagate virus stocks. Mutant adenoviruses that were propagated on 293 cells were also contaminated by AAV, suggesting that some 293 cell stocks were latently infected with AAV. Productive AAV infection requires the presence of a functional E1 region to replicate (Laughlin *et al.* 1982). 293 cells constitutively express adenovirus E1

A. WT infected cDNA probe



B. dl 312 infected cDNA probe



C. uninfected cDNA probe

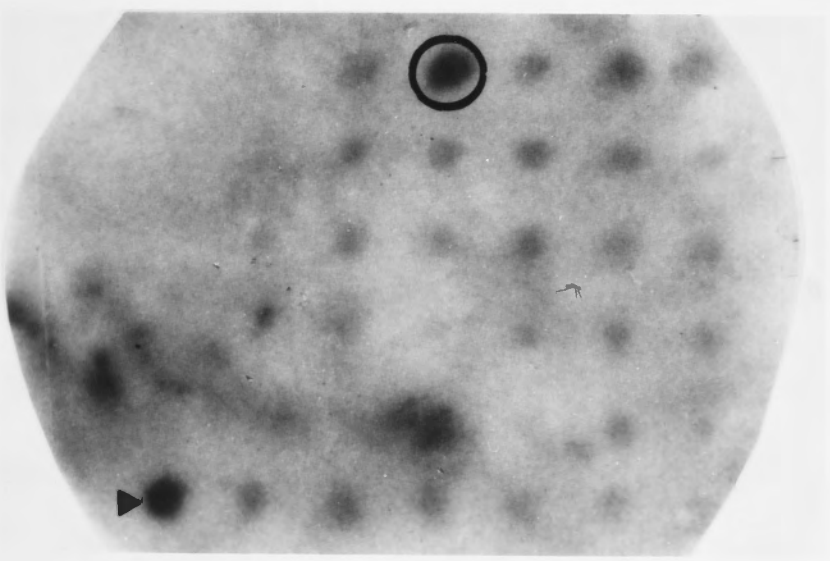


Figure 4.11 Rescreening of non-AAV clones with cDNA probes.
cDNA clones which did not show homology to AAV were rescreened with radiolabelled cDNA from A. AAV-free WT infected cells (40hrs pi), B. dl312 infected cells (40hrs pi) and C. uninfected cells.
The clone corresponding to pTD20 is circled. The positive control is indicated by the arrow head.

proteins, thus supplying the requirements for AAV rescue and replication, during mutant virus infection. Although DNA analysis did not reveal the presence of AAV in 293 cells, not all 293 stocks were comprehensively checked for the presence of AAV. There was no evidence for the latent infection of primary rat embryo fibroblasts, although AAV does exist as a latent infection in the germ line of chickens. When cells prepared from specific-pathogen-free chicks with no previous exposure to adenovirus or AAV, were challenged with purified adenovirus, AAV antigen was detected in a small number of cells (Sadasiv *et al.* 1989). Conceivably AAV may exist in the germline of rats as a provirus, however, in this series of experiments, when challenged with AAV-free adenovirus, no AAV replication was detected through Southern analysis.

AAV DNA replication is postulated to occur via a "rolling hairpin model" (Tattersall and Ward 1976). The 3' inverted palindrome terminal repeats form a hairpin structure, which functions as the primer for DNA replication. Synthesis of both plus and minus strands initiate at the 5'ends and proceed unidirectionally to the 3'end (Hauswirth and Berns 1977). Progeny SS AAV genomes are generated by strand displacement. During the co-infection of AAV and adenovirus (iu 20) in established human cell lines, AAV DNA synthesis was detected at 12 hrs pi, and progeny SS DNA was detected at 25hrs pi (Redemann *et al.* 1990). When primary human fibroblasts were infected with AAV and adenovirus, the appearance of AAV replicative intermediates, both unit length and oligomeric, was significantly delayed compared to coinfection with KB cells (Nahreini and Srivastava 1989). To investigate the extent of AAV replication at 40hrs pi, the time point at which the subtracted library was constructed, Southern analysis was performed on DNA from infected and uninfected REFs. The DNA was digested with *Sma*1, to liberate the monomer genome, as *Sma*1 restriction sites are in the inverted terminal repeats.

Sma 1-digested DNAs from 3°REFs uninfected, infected with WT, dl 312 or infected with WT+AAV, were probed with AAV. There was no hybridisation to uninfected REFs or to cells infected with AAV free WT, suggesting that REFs are not latently infected with AAV. A band corresponding to the monomer AAV genome was detected in REFs infected with dl 312, supporting previous reports that AAV requires adenovirus E1 products for productive infection (Laughlin *et al.* 1982). However, in contradiction to previous reports, there was no evidence of degradation of the AAV genome after 40 hrs of co-infection of dl 312. This lack of degradation may be a function of the different cell type used to study dl 312 co-infection, ie primary REFs in the present study as opposed to human transformed 293 cells and KB cells in the previous report (Laughlin *et al.* 1982). There was a general smear of hybridisation unique to DNA from cells infected with AAV+WT. Defective variant DNA particles, ranging in size from 3-99% of the AAV genome could account for the smear of hybridisation to DNA of lower molecular weight than the monomer genome (Hauswirth and Berns 1979, de la Maza and Carter 1980a). Variant DNA particles are generated during a high multiplicity of infection and differ in length due to internal deletions mainly in the left half of the genomic sequence, while retaining the terminal palindromic sequences (Senapathy and Carter 1984).

Hybridisation to a smear of DNA of higher molecular weight than the monomer genome in DNA from AAV and WT infected cells could indicate a number of situations. 1) AAV may contain highly repetitive sequences that cross hybridise to cellular DNA. This possibility is unlikely since there was no hybridisation to DNA from uninfected cells. 2) During the course of AAV DNA replication via the "hairpin" model and strand displacement, the terminal repeats may be incorrectly replicated, leading to the loss of the *Sma*1 sites and heterogeneous variant DNA molecules of high molecular weight. 3) Perhaps at 40 hrs pi, only replicative intermediates have been made, leading to high molecular weight AAV DNA, depending on the position of

replicative forks, and whether either terminal *Sma*1 site is double stranded (sensitive to *Sma*1 digestion) or single stranded (resistant to *Sma*1 digestion).

The fourth alternative is that AAV DNA may integrate into the cellular genome during the normal course of replication, through non homologous recombination, via the terminal repeats. The terminal repeats may be deleted at random recombination sites, which would result in hybridisation to a generalised smear of *Sma*1-digested DNA when probed with AAV. Partial or total deletions of the terminal repeats were identified in cells latently infected by AAV (Kotin and Berns 1989). Two of the AAV cDNA clones that were sequenced in chapter 3, also contained putative cellular sequences, that may have arisen through integration of AAV during DNA replication. Consequently, transcription from AAV promoters may continue through the adjacent "cellular" sequences, and the resulting chimeric RNA species could have been cloned in the original cDNA library. Alternatively, the association between the cellular sequences and the AAV sequences may be a technical artifact, as there were no terminal repeat sequences associated with the junctions of AAV and cellular sequences, and integration is believed to take place via recombination involving the terminal sequences (Cheung *et al.* 1980).

Other DNA viruses integrate into the cellular genome during productive infection. Large amounts of adenoviral DNA are associated with cellular DNA during productive or semi-permissive growth, and even when viral growth is prevented (Schick *et al.* 1976, Tyndall *et al.* 1978). Vogel *et al.* (1986) used an *in situ* technique to demonstrate the integration of adenovirus DNA into a limited number of chromosomes, early in productive infection. Thus it is likely that the mechanism of integration before replication of viral DNA is shared between AAV and adenovirus.

The general smear of hybridisation unique to DNA from cells infected with AAV and WT after probing with AAV may not be solely due to concatemeric strand displacement of SS AAV progeny or recombination with the cellular genome. AAV replication may promote homologous reciprocal recombination between episomal and integrated forms of AAV, resulting in possibly integrated, expanded and contracted forms of the AAV genome. Hauswirth and Berns (1979) found that short defective DNA molecules, generated during productive infection were crosslinked at or near one terminus and some were generated by a template strand switch. However statistical investigation of partial base pairing potential in the recombination of SV40 DNA in eukaryotic cells by Savageau *et al.* (1983) found no statistical significance in patchy homology within the recombining region of SV40. Thus, the generation of variant AAV genomes due to homologous recombination at short stretches of homology between integrated and episomal AAV, must be considered cautiously.

The AAV clones identified in chapter 3, mapped between 75.8 and 91 mu of the AAV genome. The clones are clustered to the 3' region of the AAV genome, probably due to the method of synthesis of the cDNA used in library construction. During the preparation of the cDNA library, poly A^+RNA from WT infected cells, was primed with oligo (dT), which would anneal to terminal poly A^+ tracks before reverse transcription to synthesise cDNA. Reverse transcription would initiate at the 3'regions of RNA transcripts, and the extent of progression towards the 5'region would depend on the efficiency of the enzyme and reaction conditions. One would expect cDNAs which represent the 3' regions of the original RNA transcripts, and an under-representation of the 5' regions. Degradation of cDNA during subtractive hybridisation and hydroxylapatite chromatography, as evidenced by the low molecular weight cDNA inserts characterised in chapter 3, may have contributed to the appearance of "clustered" cDNA clones.

The kinetics of AAV transcription was investigated by probing RNA isolated at different times from cells coinfected with WT+AAV, with the AAV clones. The clones were able to detect all six major transcripts of AAV in total cytoplasmic RNA from WT+AAV infected cells. The AAV RNA species are transcribed from 3 promoters. The RNA species of 4.2kb and its spliced form of 3.9kb are transcribed from the promoter at 5mu. The transcription from the promoter at 19mu results in RNA of 3.6kb and 3.3kb (spliced). The RNA responsible for structural proteins, of 2.6 and 2.3kb (spliced) are transcribed from the promoter at 40mu (Green and Roeder 1980, Laughlin *et al.* 1979, Green *et al.* 1980).

Northern analysis of total cytoplasmic RNA from WT and AAV infected cells showed that AAV transcription was detected within 30 min of coinfection. The expression of this RNA of approximately 5kb increased at 10hrs pi and remained constitutively high through the rest of infection. RNA of 4.2 and 3.9kb, coding for replication and regulation proteins were detected at 8 hrs pi, and the level of transcription did not increase during infection. The 2.3 and 2.6kb RNA, responsible for structural proteins were detected at 8 hrs, increasing in levels at 10hrs pi and remaining constant during infection. Hybridisation with some clones revealed a novel RNA transcript of 3.3kb induced at 30-40hrs pi, suggesting that transcription from the promoter at 19 mu increased significantly at 30-40hrs from a low constitutive level. A previously undescribed transcript of 1kb was also detected at 10hrs.

It is not clear why the AAV clones, which map closely together on the AAV genome, show two distinctly different hybridisation patterns. Clones 1,3 and 9 hybridised to bands in a different pattern to clones 4 and 5, differing mainly in relative band intensities. The difference in band intensity may be a technical artifact, perhaps due to poor transfer of RNA to the nylon membranes. However, gels were restained after transfer to monitor the transfer efficiency. The alternative hypothesis is that variable

amounts of AAV DNA have contaminated the RNA stocks. The procedure of RNA extraction relied on the centrifugation of undamaged nuclei to remove DNA. In hindsight, centrifugation of all RNA solutions through cesium chloride during preparation, as described for the isolation of nuclear RNA (section 2.2.4) would be necessary to remove DNA contamination. To verify the presence of DNA in the RNA samples, the membranes need to be treated with RNase-free DNase and be re-exposured to autoradiographic film. Once the presence of DNA is established the samples could be treated with DNAse, and the Northern analyses repeated. If the difference in hybridisation pattern is not accounted for by technical artifact, perhaps the different clones show sufficient homology to other parts of the AAV genome to allow increased hybridisation to different transcripts. This was investigated by comparing the clones to the AAV genome in the form of a dot matrix. The clones were not significantly homologous to other parts of the genome.

The original subtracted cDNA library was re-examined to identify cellular sequences induced by E1A in the presence or absence of AAV. Although AAV sequences were extensively dispersed throughout the differentially screened clones from the original subtracted cDNA library, it was possible to isolate a cellular cDNA clone that showed hybridisation to radiolabelled cDNA synthesized from AAV free WT infected cells and cDNA from dl 312 infected cells out of 77 clones. It is possible that other cellular transcripts in low abundance, were not isolated after rescreening because cDNA probes were not enriched for rare transcripts by subtraction. Several investigators have found that specific cDNA clones were not detected in plasmid libraries if 0.05% to 0.2% of their probes did not contain the gene of interest (Dworkin and David 1980, Crampton *et al.* 1980). Rare transcripts (0.001-0.005% abundance) would be undetectable with unfractionated cDNA probes, therefore the non-AAV clones should have been rescreened with enriched cDNA probes (Davis *et al.* 1984a). The isolated cellular clone was designated pTD20 and is analysed further in chapter 5.

Summary

AAV is a defective parvovirus, dependent on the presence of helper viruses for productive infection. AAV infiltrated the adenovirus stocks used to construct a subtractive cDNA library, enriched for E1A induced cellular sequences. The presence of AAV sequences amongst the cDNA clones indicates that the library was enriched for E1A-induced sequences, as dl 312 infection does not support AAV replication and transcription in REFs. AAV replication in REFs involved the synthesis of replicative intermediates, concomitant with possible recombination between episomal AAV and cellular DNA, resulting in integration, during co-infection with adenovirus. At 40hrs pi AAV DNA strands of variable length (of both higher and lower molecular weight than the monomer genome) were generated, although higher molecular weight species were not encapsidated into virions.

Transcription of AAV was detected by using AAV clones isolated from the subtracted library, within the first 8 hrs after co-infection with adenovirus. By 10 hrs after co-infection, transcription from all three promoters was detected. Transcription from the p19 promoter increased at 30-40 hrs.

To isolate cellular genes influenced by adenovirus in the absence or presence of AAV, the differentially screened clones, isolated from the subtracted library, as described in chapter 3, were rescreened with cDNA synthesized from uninfected, WT infected and WT+AAV infected cells. One clone (pTD20) from 77 clones, hybridised to all cDNA probes and was further studied in chapter 5.

Chapter 5

Expression of two cellular genes during adenovirus infection

Introduction

As discussed in chapter 4, adenovirus induces the replication of AAV, and AAV replication affects the replication and transcription of adenovirus. In the absence of AAV, adenovirus E1A gene products activate transcription from viral and cellular promoters. In this chapter, a cellular cDNA insert, isolated from a subtracted cDNA library enriched for E1A-induced sequences, was characterised by sequencing, Southern and Northern analysis. The cDNA clone was induced by adenovirus and uninfluenced by AAV. The clone was highly similar to B2 repetitive sequences, which have enhanced transcription in transformed cells.

Adenovirus induction of other cellular genes, thought to influence the intracellular environment towards transformation, was also studied in the presence or absence of AAV by Northern analysis. Increasing expression of the mRNA for ubiquitin, an important protein in the ATP-dependent degradation pathway for damaged, denatured or foreign proteins (Ciechanover *et al.* 1984), was found to be the result of both transactivation and post transcriptional regulation mediated by E1A and E1B during adenovirus infection. Increased levels of ubiquitin may ultimately lead to enhanced degradation of viral proteins, inducing the establishment of a latent infection and possible adenovirus-induced transformation.

Isolation of a cellular gene induced by E1A during adenovirus infection

The subtracted cDNA library, enriched for sequences induced by E1A, as described in chapter 3, was re-examined to isolate cellular sequences, uninfluenced by AAV, and induced by adenovirus infection. As discussed in chapter 4, one clone from 77 differentially screened clones hybrised to cDNA probes made from AAV-free WT infected cells and uninfected cells, and not to AAV. This clone, designated pTD20, was analysed by sequencing, Northern and Southern analyses.

Sequence analysis

The 163 bp insert of pTD20 was sequenced using the pUC sequencing kit as described in section 2.2.9. When the sequence (fig 5.1) was compared to the sequences listed in the EMBL databank, using the algorithm of Wilbur and Lipman (as discussed in chapter 3), there was significant similarity to B2 repetitive sequences (EMBL no. 18853) at the nucleotide level (similarity 75%). A consensus B2 rodent sequence was determined by comparison of 3 individual mouse, 2 rat and 2 hamster copies of the sequences (Krayev *et al.* 1982). The alignment of TD20 to the consensus rodent B2 sequence is shown in fig. 5.2.

Southern Analysis

DNA isolated from uninfected cells, was digested with *Pst*1 or *EcoR*1/*BamH*1 before electrophoresis through agarose and Southern transferred to a nylon membrane (section 2.2.6). The membranes were probed with ^{32}P labelled TD20. The intense smearing, obvious in fig 5.3, was indicative of a highly repetitive element with dispersed organization within the genome.

Northern analysis of pTD20

Initially, RNAs from uninfected and WT infected cells were denatured and transferred to nylon membranes before probing with pTD20, as described in section 2.2.4. As shown in figure 5.4.a, the transcripts of pTD20 ranged in size from approximately 150 to 400bps, and were constitutively expressed in uninfected cells. There was a 3-7 fold increase in the level of transcription 40 hours post infection with WT (fig. 5.4.b). The membranes were reprobed with α-tubulin to correct for RNA loading. The induction was unaffected by AAV coinfection and the transcript was similarly induced when serum deprived cells were infected with WT, (demonstrated in fig. 5.5), suggesting that the induction of pTD20 was due to WT virus infection. To identify the region of adenovirus responsible for the induction, RNA isolated from cells

Figure 5.1 Sequence of TD20, 163bps

```
                                                                    50
TGAACGACTG  ACTGACTGCT  CTTCCTGAGG  TCCTGAGTTC  AAATCCCAGC
                                                                   100
ACCACGATGT  GTGCTGACAC  ATCTGAGTGA  GGTCTGATGC  CTCTTCTGTG
                                                                   150
TGTCTGAAGA  CAGTGACAGT  GTACTCACAT  ACATGAAATA  AATAAATCTT

TAAAAAAAAA  AAA
```

Figure 5.2 Alignment of the consensus rodent B2 sequence (192bps) with TD20 (163bps)
: nucleotide homology

```
X         10         20         30         40         50
GGGCTGGAGAGATGGCTCAGTGGTTAAGAGCACCTGACTGTCCTTCCGAAGGTCCTGAGT   B2
                       : ::    :::::::  :::::  ::::::::::::
                     TGAACGACTGACTGACTGCTCTTCCTGAGGTCCTGAGT   TD20
                     X         10         20         30

          70         80         90         100        110
TCAATTCCCAGCAACCACATGGTGGCTCACAACCATCCGTAATGAGATCTGATGCCCTCT   B2
:::: :::::::: :   :::   ::: :::     :  :   :::: :::::::::    :
TCAAATCCCAGCACCACGATGTGTGCTGACACATCTGAG---TGAGGTCTGATGCCTCTT   TD20
 40         50         60         70             80         90

          130        140        150        160        170
TCTGGAGTGTCTGAAGACAGCTACAGTGTACTTACATATAATAAATAAATAAATCTTTAA   B2
      ::::::::::::::  :::::::::: ::::: :  ::::::::::::::::::
CTGT--GTGTCTGAAGACAGTGACAGTGTACTCACATACATGAAATAAATAAATCTTTAA   TD20
      100        110        120        130        140        150

           X
AAAAAAAAAAAA                                                   B2
::::::::::
AAAAAAAAAA                                                     TD20
      160X
```

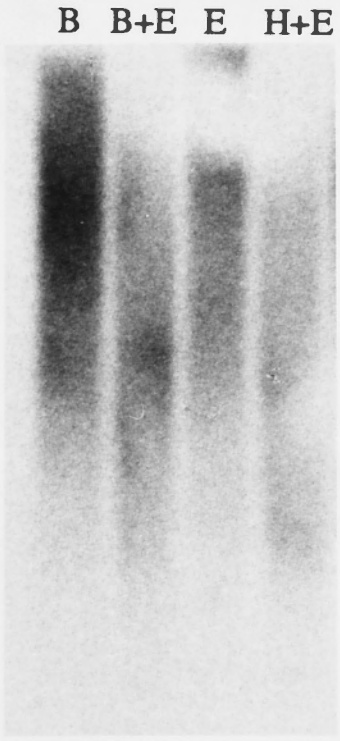
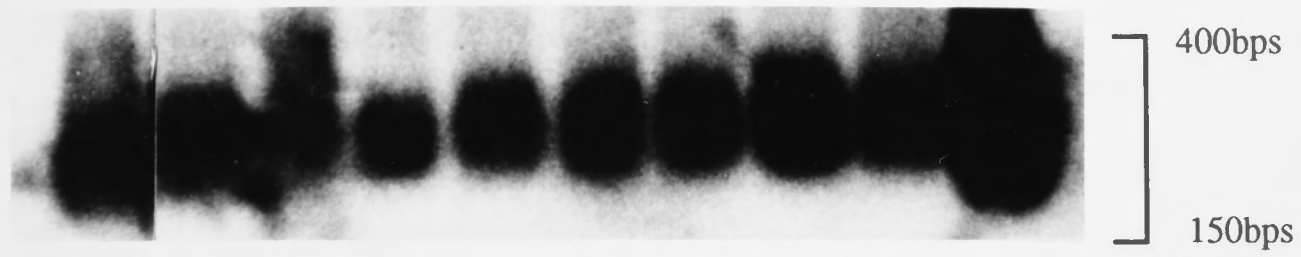


Figure 5.3 Southern analysis of TD20.
10μg DNA from uninfected 3°REFs was digested with *Bam*H1 (B), *Bam*H1 and *Eco*R1 (B+E), *Eco*R1 (E) and *Eco*R1 with *Hinc*II (H+E), electrophoresed, transferred to a nylon membrane and probed with TD20.

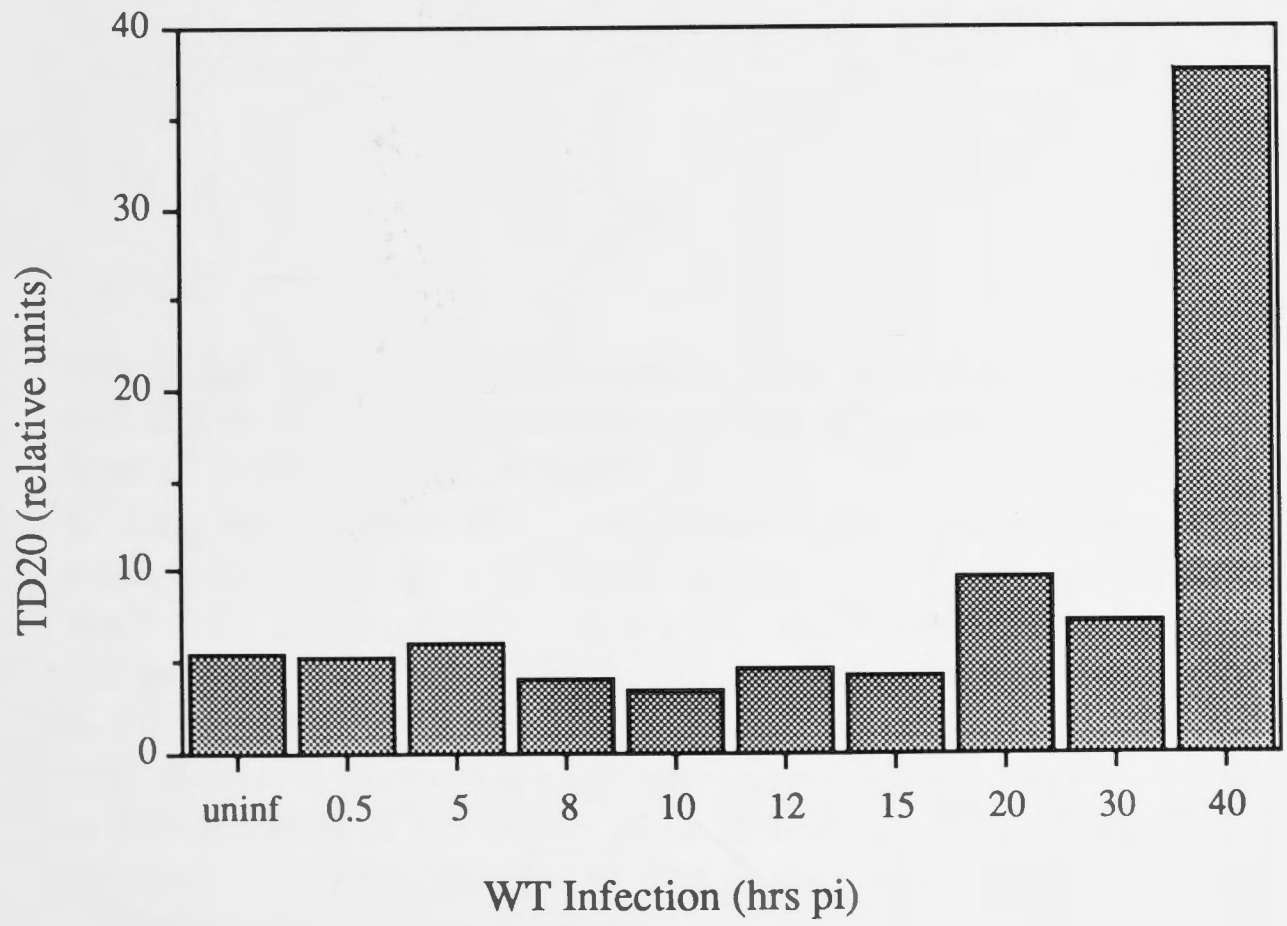
Figure 5.4. Analysis of induction of TD20 by adenovirus.
a. 20μg total cytoplasmic RNA was isolated from uninfected (un) and WT- infection cells (at indicated times after infection), denatured and transferred to nylon membranes, before probing with TD20.
b. Densitometric comparison of levels of cytoplasmic TD20 during infection. ▩ TD20 mRNA (relative units)

a. TD20 probe

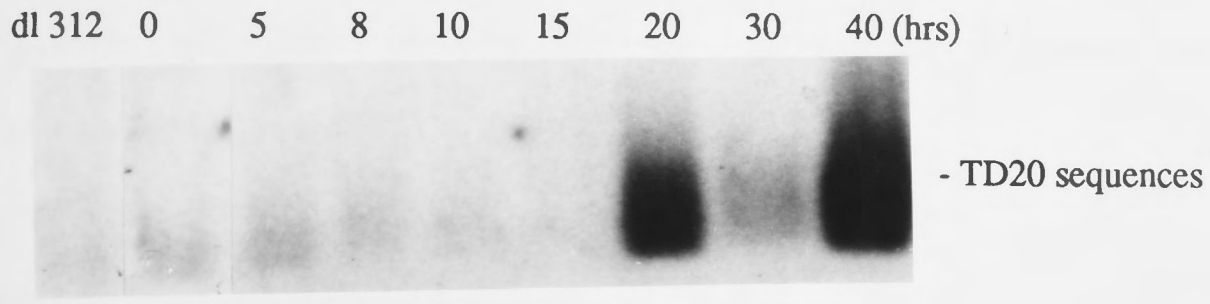


α-tubulin reprobe

b. level of cytoplasmic TD20 mRNA during WT infection



a. TD20 mRNA levels during WT+AAV infection



b. TD20 mRNA levels during WT infection of serum starved cells

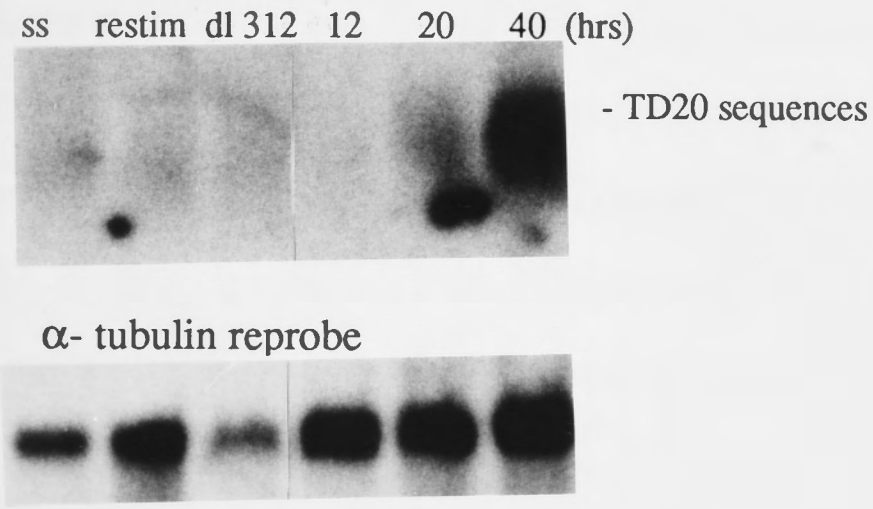


Figure 5.5 Effect of co-infection of cells with AAV and WT, and effect of serum deprivation during WT infection, on the level of TD20 cytoplasmic mRNA.

a. 20μg cytoplasmic RNA was isolated from uninfected (un) cells, cells with dl 312 (40hrs pi) and cells infected with AAV+WT at various times after infection, Northern transferred and probed with TD20 cDNA.

b. 20μg cytoplasmic RNA was isolated from serum deprived cells that were uninfected (ss), restimulated with serum (restim) or infected with dl 312 (40hrs pi) or WT at various times during infection, Northern transferred and probed with TD20 cDNA, before reprobing with α-tubulin.

infected with mutant viruses, was Northern transferred and probed with TD20. As shown in fig. 5.6., none of the infections with mutant viruses resulted in a dramatic increase in TD20 expression as was detected in WT-infected cells. This result suggests that the expression of all genes from the adenovirus early region is required for efficient transcriptional activation of TD20.

Control at the post transcriptional level

Nuclear run on experiments (section 2.2.3) were used to investigate whether the adenovirus-induced increase of low molecular weight RNAs transcribed by B2 repetitive sequences was due to an increased rate of transcription or post transcriptional changes. Nuclei were isolated from infected cells, nascent RNA chains were elongated by RNA polymerase, in the presence of a radiolabelled ribonucleotide, and used to probe cDNA-containing plasmids immobilized on nylon membranes. As demonstrated in fig 5.7 the TD20 repetitive sequence was actively transcribed in cells that were uninfected. TD20 sequences were also actively transcribed in cells infected with WT adenovirus and with mutants lacking a functional complete E1A, ie 12S, 13S, 12S PM961 and hr 3. Transcription was not affected by the lack of a functional E1B region, as shown by cells infected with the E1B mutants, dl 338, dl 337.

In each case, the rate of transcription of TD20 in cells infected with most of the mutant viruses was at least double that of the uninfected cells. The WT virus was the most efficient at increasing the rate of TD20 transcription by 6 fold. The 12S and 13S viruses induced an approximate 4 fold increase. Viruses which contained mutations within the 289aa protein, the putative CD3 region, eg hr 3, induced TD20 transcription by 2 fold. The virus that contained a mutation within the CD2 region within the 243aa protein (12Spm961) induced TD20 expression 5 fold, while the virus that contained a mutation within the CD2 region in both E1A proteins (E1Apm928) exhibited WT induction. Thus it appears that mutations within the CD2 region do not affect the positive induction ability of adenovirus, and mutations within the CD3 region diminish

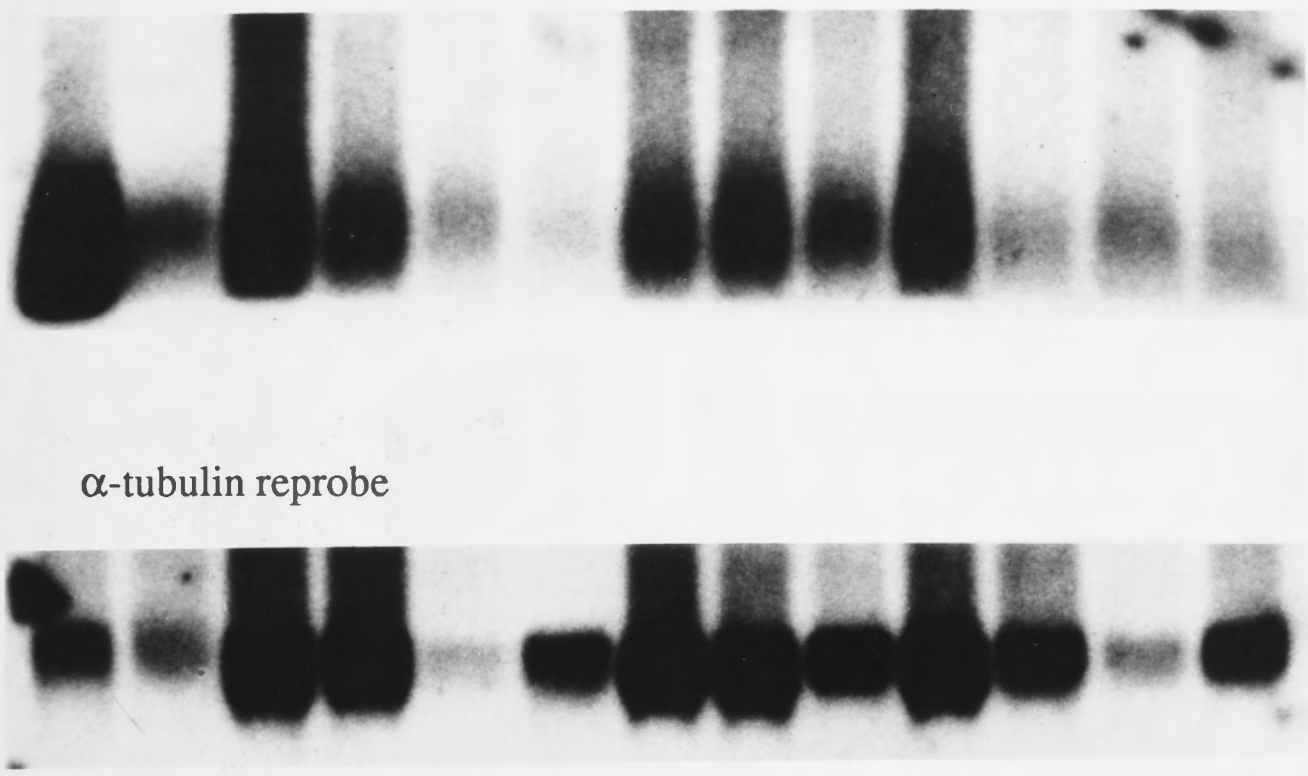
Figure 5.6 Analysis of TD20 cytoplasmic mRNA levels after infection with mutant viruses.

a. 20μg total cytoplasmic RNA from cells uninfected (un), or infected with WT, dl 312, 13S, 12S, hr3, 12SPM961 (pm961), E1Apm928 (pm928), dl 313, dl 337, dl 338, dl 327, or dl 808, 25iu/cell, 40hrs pi, was transferred to a nylon membrane and probed with labelled TD20, before reprobing with α-tubulin.

b. Densitometric comparison of TD20 mRNA levels in the cytoplasm of cells infected with different adenovirus mutants

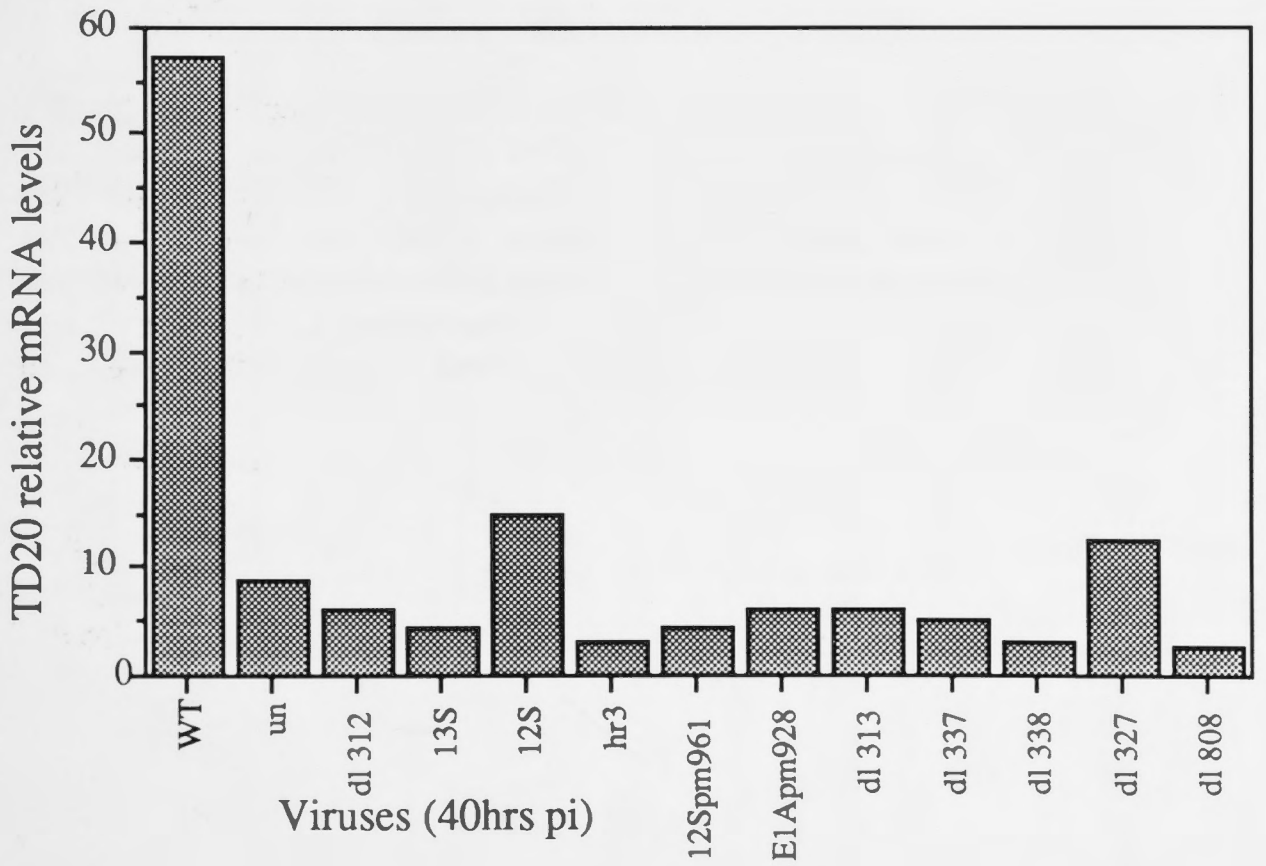
▩ pTD20 mRNA

a. TD20 mRNA levels 40 hrs after mutant virus infection



α-tubulin reprobe

b. TD20 cytoplasmic mRNA levels 40 hrs after mutant virus infection



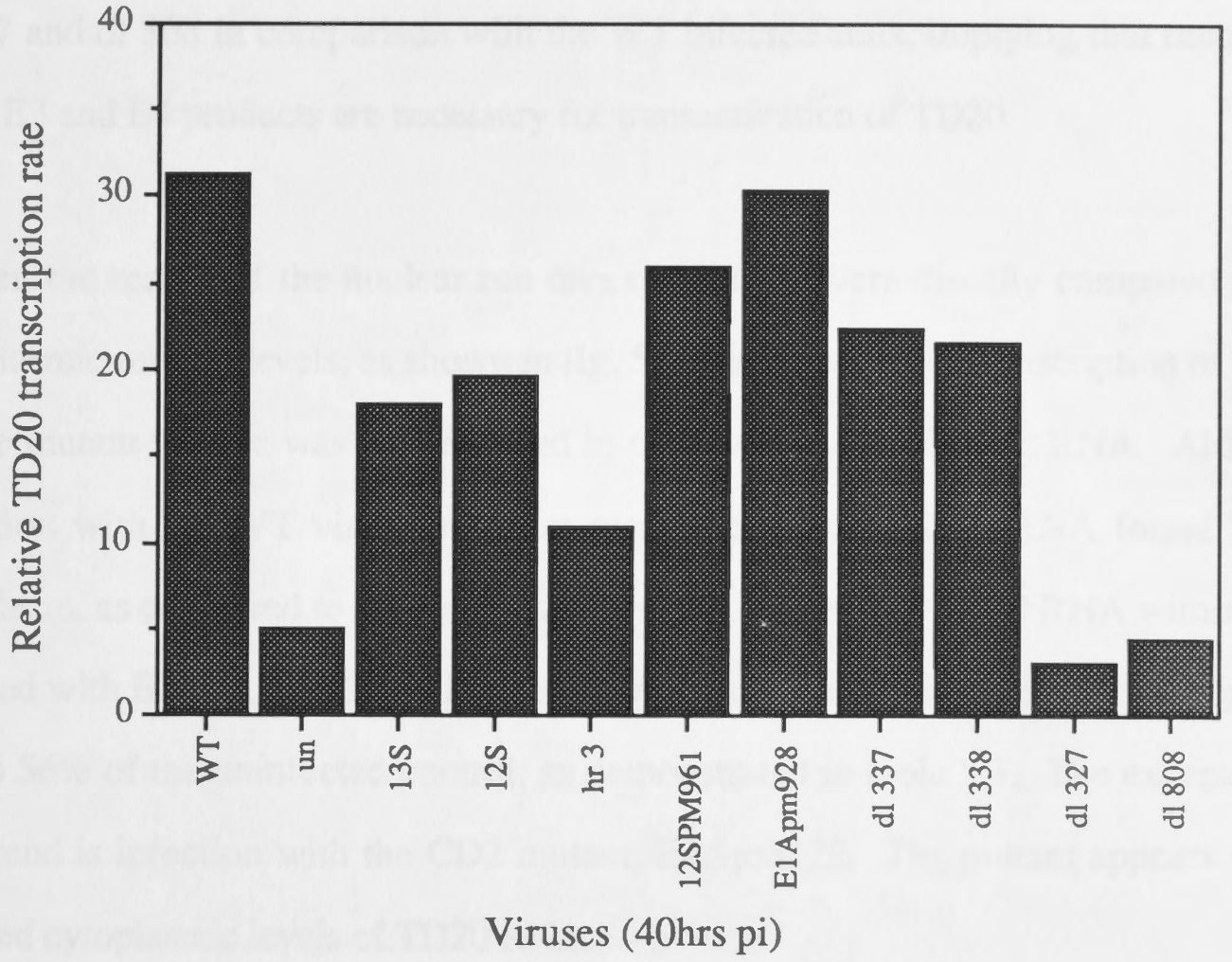


Figure 5.7. Effect of Ad mutant infection on transcription rate of TD20. Nuclei were isolated from cells 40 hrs after infection with WT, 13S, 12S, hr3 12Spm961, E1Apm928, dl 337, dl 338, dl 327, dl 808 (25iu) and uninfected (un) cells. Nascent RNA chains were elongated in the presence of a radiolabelled nucleotide and used to probe TD20 plasmid immobilized on a membrane.

■ TD20 transcription level

the transactivation of TD20 by one third. The E1B mutants increased transcription by 4.5 fold. An unusual result was obtained during infection with E3 (dl 327) and E4 (dl 808) mutant viruses. The transcription rate of TD20 was reduced during infection with dl 327 and dl 808 in comparison with the WT infected cells, implying that functional E1A, E3 and E4 products are necessary for transactivation of TD20.

When the results of the nuclear run on experiments were directly compared to the cytoplasmic mRNA levels, as shown in fig. 5.8, the induction of transcription of TD20 by the mutant viruses was not reflected in the level of cytoplasmic RNA. Although infection with the WT virus showed a clear increase of TD20 RNA found in the cytoplasm, as compared to the uninfected control, the level of TD20 RNA within cells infected with E1A, E1B, E3 and E4 mutant viruses was reduced to levels ranging from 8% to 56% of the uninfected control, as demonstrated in table 5.1. The exception to this trend is infection with the CD2 mutant, E1Apm928. The mutant appears not to affected cytoplasmic levels of TD20 RNA.

Thus the increase of low molecular RNA transcribed from TD20 repetitive sequences at 40 hours after infection with WT, was due to an increased transcription rate of the genes. Infection with adenovirus mutants has demonstrated the need for functional viral genes from the early region of adenovirus to assist in increasing mRNA stability or transport from the nucleus to the cytoplasm, for mutations within the early region result in decreased levels of RNA in the cytoplasm.

Effect of adenovirus infection on ubiquitin expression

Analysis of ubiquitin RNA levels during adenovirus infection

Total cytoplasmic RNA from uninfected cells and cells infected with WT virus at various times after infection, was Northern transferred to nylon membranes, as described in section 2.2.4, probed with the human ubiquitin cDNA and reprobed with

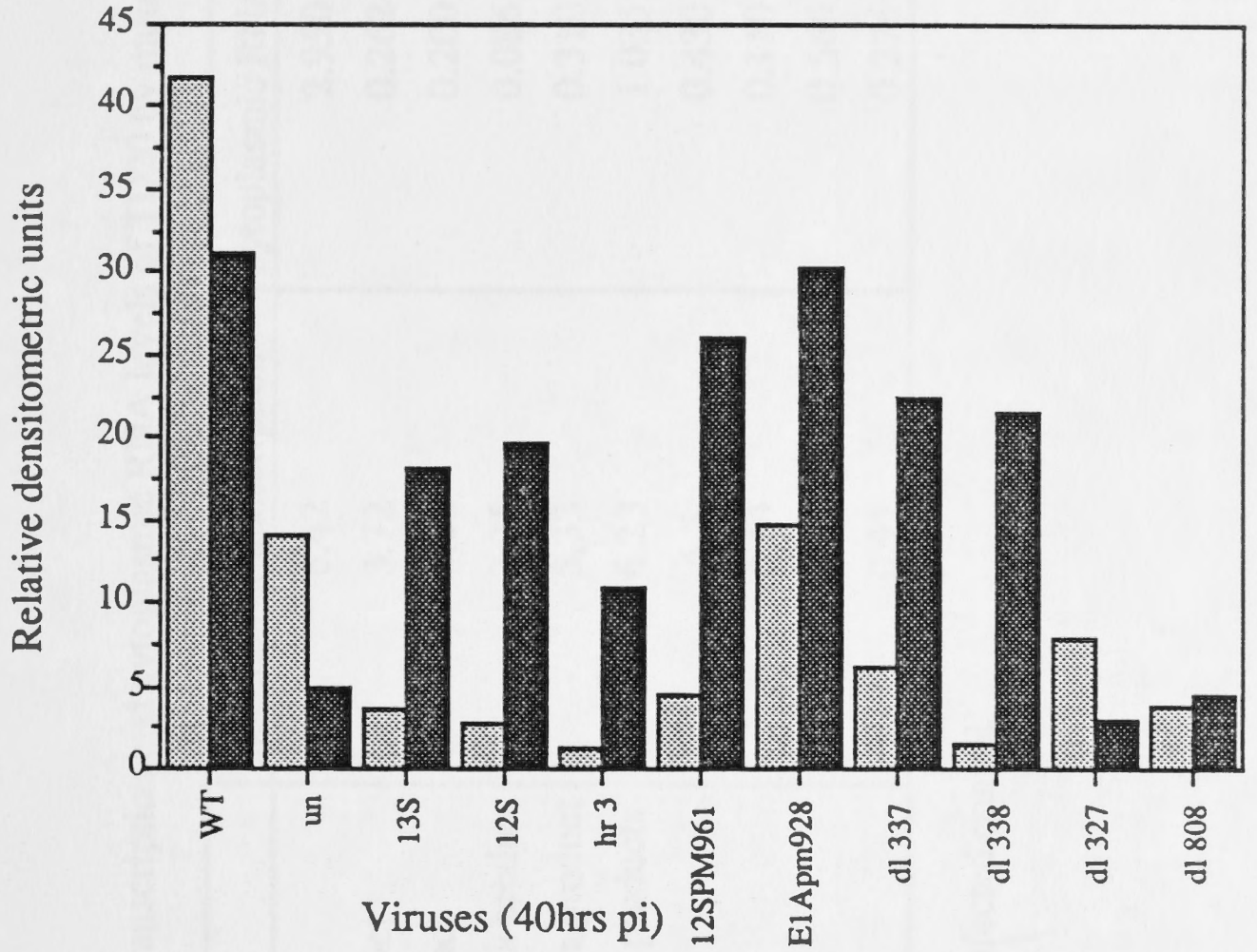


Figure 5.8 Relative levels of mRNA and transcription rates of TD20 during Ad mutant infection

pTD20 mRNA levels

TD20 transcription level

Table 5.1 Induction of the rate of transcription and cytoplasmic RNA levels of TD20 by mutant viruses

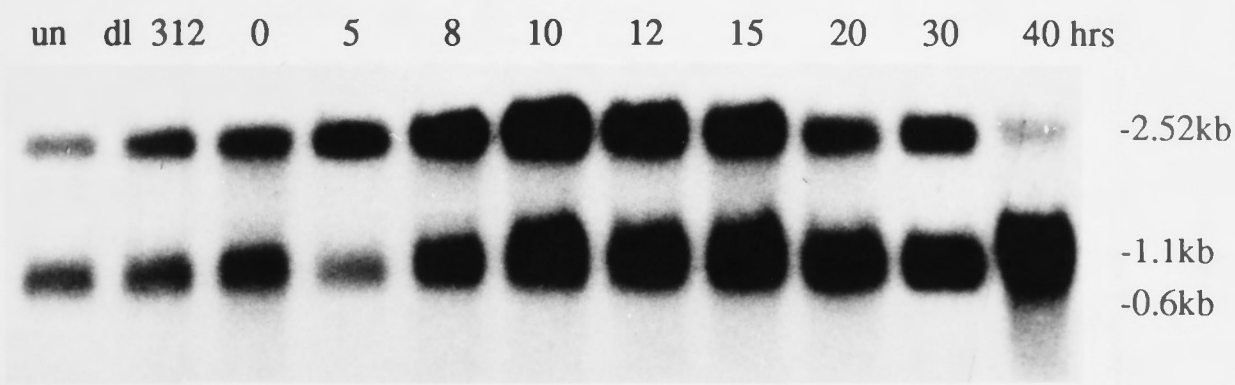
Viruses	Mutation	induction of transcription[AB]	cytoplasmic RNA levels[AC]
WT		6.42	2.950
13S	only 289aa E1A product	3.72	0.262
12S	only 243aa E1A product	4.02	0.200
hr 3	CD3 mutation in 289aa product	2.25	0.086
12Spm961	CD2 mutation in 243aa product	5.33	0.310
E1Apm928	CD2 mutation in E1A products	6.23	1.035
dl 337	no E1B 21S protein	4.5	0.430
dl 338	no E1B 55S protein	4.44	0.110
dl 327	no E3	0.6	0.560
dl 808	no E4	0.91	0.276

[A] Level of induction compared to uninfected control
[B] Data from figure 5.7
[C] Data from figure 5.6

α-tubulin. As shown in fig 5.9, the ubiquitin probe hybridised to two major transcripts of 2.52kb and 1.1kb, and a minor transcript of 0.6kb. These sizes agree well with the rat and human transcripts described previously (Latchman *et al.* 1987, Wiborg *et al.* 1985). The major RNAs of 2.52kb and 1.1kb, code for tandem repeats of the 76aa protein transcribed from the polyubiquitin genes Ub C and Ub B, respectively and the minor RNA of 0.6kb codes for a single-copy-ubiquitin fusion protein transcribed from Ub A genes (Wiborg *et al.* 1985, Baker and Board 1987, Lund *et al.* 1985). During the course of adenovirus infection, levels of Ub B cytoplasmic RNA increased, while levels of Ub C cytoplasmic RNA increased then decreased. This trend is shown in figure 5.10. The difference between Ub B and Ub C RNA levels is most dramatic at 40hrs post infection, (an 8 fold difference). There is an 1.6 fold difference between Ub B and Ub C mRNA in uninfected cells, while dl 312 infected cells contained equivalent amounts of Ub B and Ub C mRNA. This result implies that adenovirus infection increases the level of Ub C mRNA during the early stage of infection, followed by a gradual reduction of Ub C RNA. At the same time, Ub B RNA gradually increases during the course of infection.

To investigate whether the preferential accumulation of Ub B RNA was due to post transcriptional regulation, nuclear and cytoplasmic RNA was isolated at various times during infection, Northern transferred and probed with ubiquitin cDNA. As shown in fig. 5.11.a, the cytoplasmic RNA levels of Ub B and Ub C were similar until 20hrs post infection, when Ub B levels increased significantly. Ub B nuclear RNA levels were always increased in comparison to Ub C nuclear levels (fig 5.11.b), and there was no major increase of expression of either nuclear Ub B or Ub C mRNA levels. This result suggests that Ub B is transcribed at a greater rate than Ub C, although this is only reflected at the cytoplasmic level during adenovirus infection. Perhaps the transport of Ub B mRNA to the cytoplasm is normally repressed, and this repression is relieved during adenovirus infection. Alternatively, perhaps cytoplasmic Ub B mRNA is stabilised during adenovirus infection.

Ubiquitin probe



α-tubulin reprobe

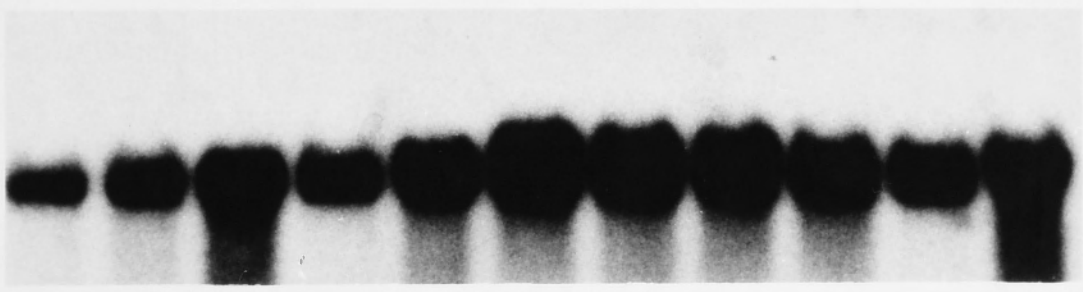


Figure 5.9 Expression of ubiquitin during WT infection
20μg total cytoplasmic RNA was isolated from uninfected (un) cells, cells infected with dl 312, and cells infected with WT at various times after infection. After transfer to a nylon membrane, the RNA was probed with ubiquitin cDNA, then reprobed with α-tubulin.

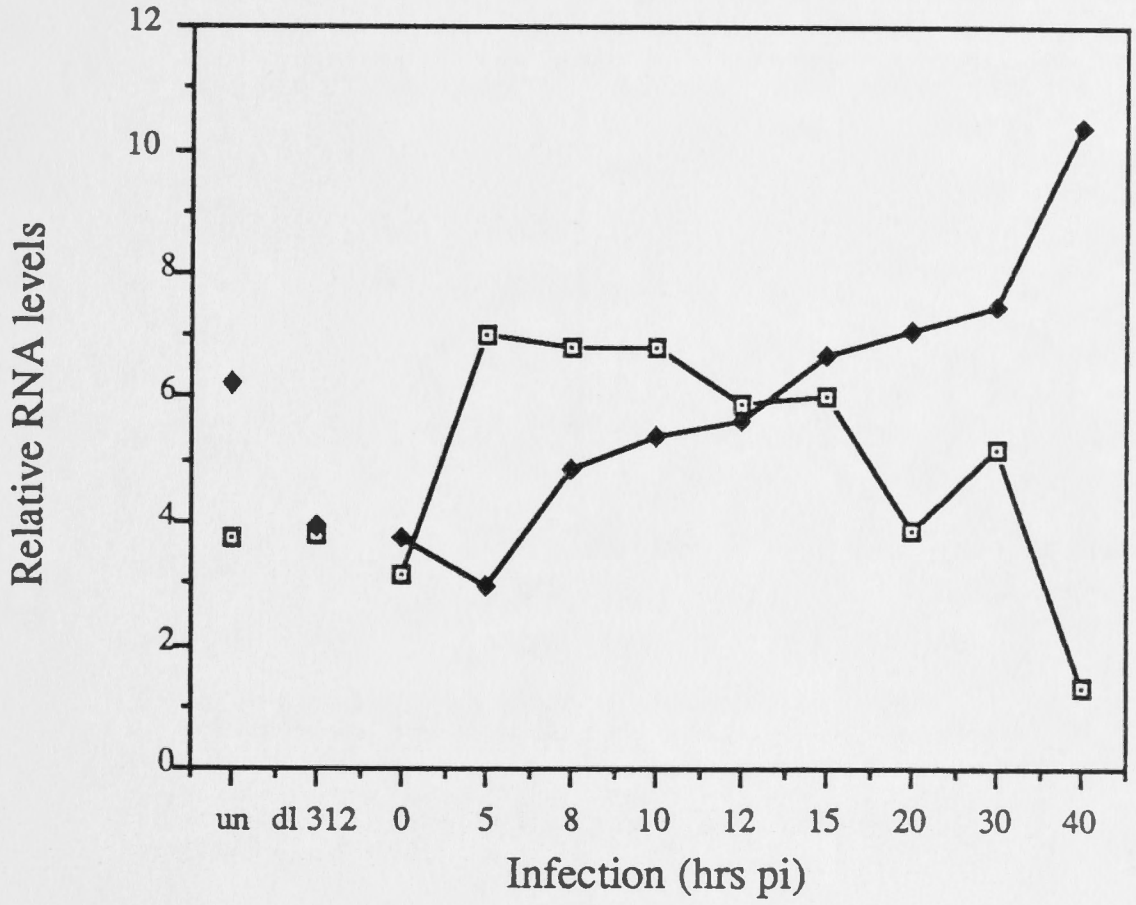


Figure 5.10 Levels of cytoplasmic ubiquitin mRNA during WT infection

—□— Ub C cytoplasmic mRNA

—◆— Ub B cytoplasmic mRNA

Figure 5.11 Expression of ubiquitin during adenovirus infection.
a. Levels of Ub B and Ub C mRNA in the cytoplasm during infection with WT at 0, 20, 40 hrs pi, and infection with dl 312 (40hrs pi).
i. Northern analysis
ii. Relative comparisons

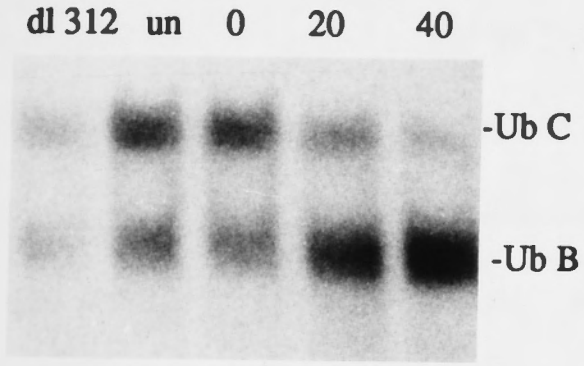
—▪— Ub C cytoplasmic mRNA
—◆— Ub B cytoplasmic mRNA

b. Levels of nuclear Ub B and Ub C mRNA during WT infection.
i. Northern analysis
ii. Densitrometric comparisons

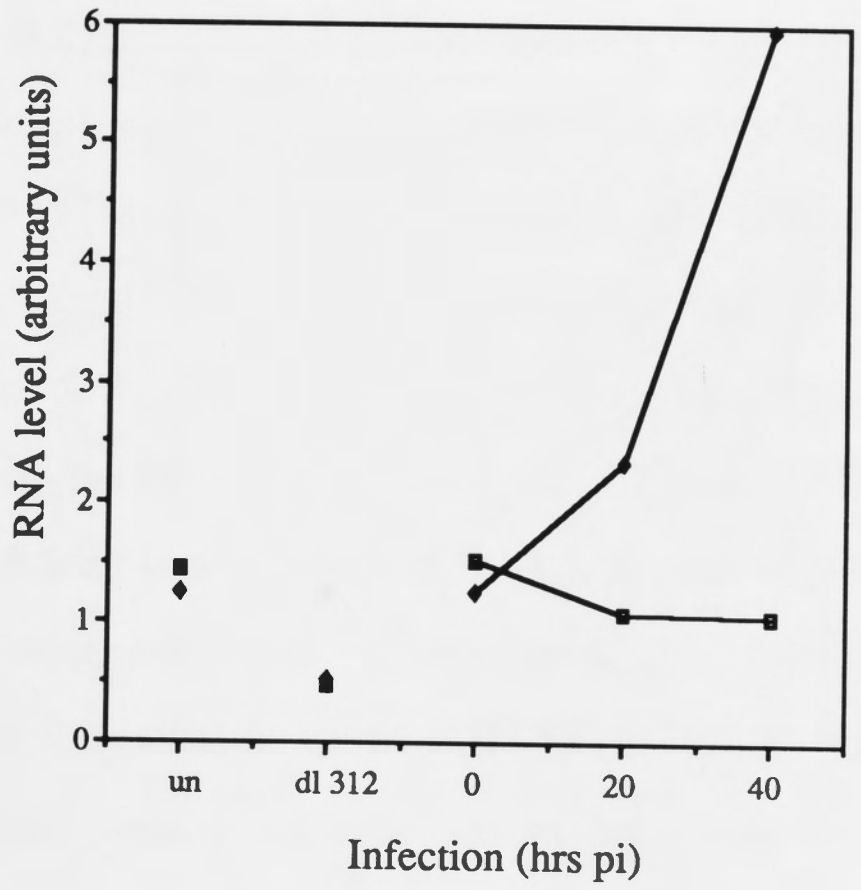
—■— Ub C nuclear mRNA
—◇— Ub B nuclear mRNA

a. Induction of cytoplasmic Ubiquitin mRNA during Ad infection

i. Northern

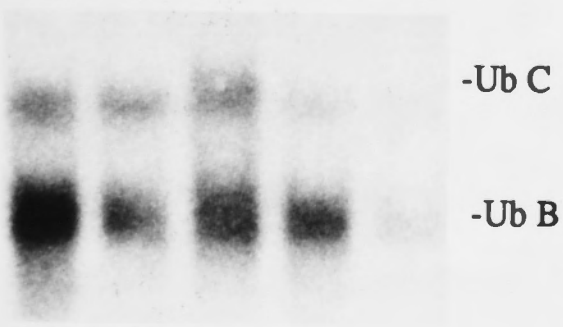


ii. Densitometric comparison.

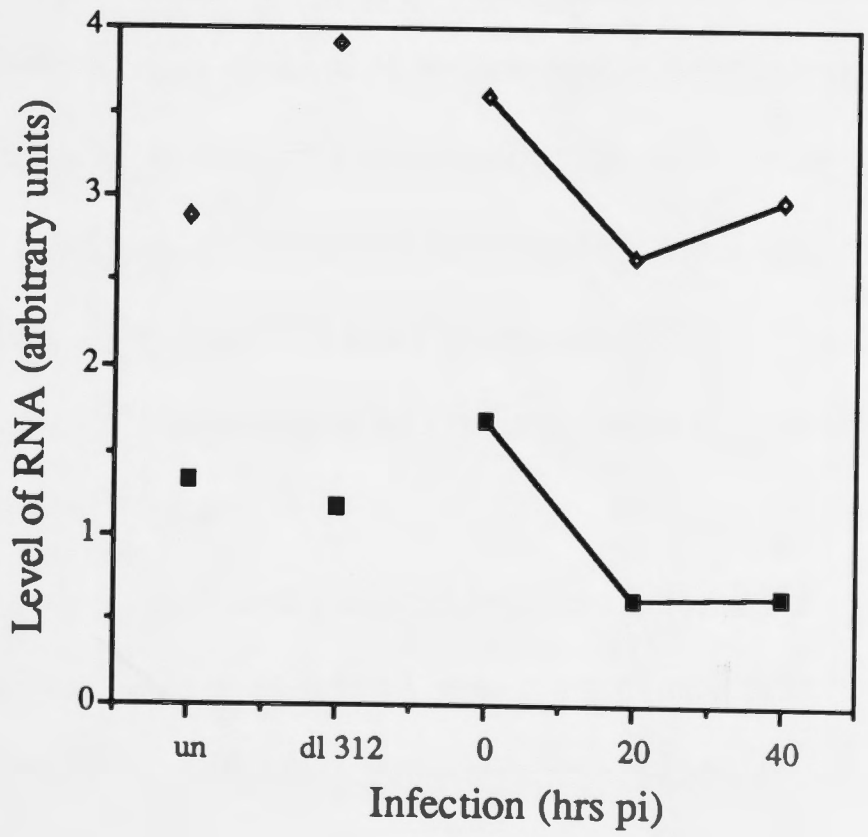


b. Induction of nuclear Ubiquitin mRNA during Ad infection

i. Northern



ii. Densitometric comparison.



To identify the regions of adenovirus responsible for the induction of Ub B RNA levels, nuclear and total cytoplasmic RNA isolated from cells infected with adenovirus mutants for 40hrs, was Northern transferred and probed with the ubiquitin cDNA. As shown in fig.5.12.a., the induction of cytoplasmic Ub B RNA over Ub C (2.4 fold) was most prominent during WT infection. Uninfected and dl 312-infected cells contained comparable levels of Ub B to Ub C. Infection with the other E1A mutants, 12S and 13S, showed an increase of 1.2 and 1.5 fold of Ub B levels, respectively, when compared to Ub C levels. Infection with the E1B mutant, dl 313, showed a similar increase, while infection with the E3 and E4 mutants (dl 327 and dl 808) resulted in an increase of 2.3 and 1.8 fold, respectively, more closely resembling the induction of Ub B by WT infection. This experiment suggests that the selective relative increase of Ub B mRNA levels in the cytoplasm is dependent on a functional E1A and E1B unit, and the presence of a functional E3 and E4 unit is not an absolute requirement.

In this particular experiment, the level of cytoplasmic ubiquitin RNA was extraordinarily high in uninfected cells. Previous experiments have revealed low levels of cytoplasmic ubiquitin RNA in uninfected cells when compared to levels in WT infected cells. The high levels within uninfected cells may reflect a variable Northern transfer (although this was not obvious after α-tubulin reprobing). Alternatively, perhaps the flasks of uninfected cells were uniquely exposed to an environmental stress, eg. pH changes within the media or temperature differences, leading to an induction of stress proteins, including ubiquitin. All cells were handled in the same way within each experiment, so a definitive explanation of the increase in cytoplasmic ubiquitin levels in uninfected cells is not possible.

The rate of ubiquitin transcription was not significantly altered from the uninfected level during virus infections. The level of nuclear Ub B mRNA was consistently at least 2 fold higher than the nuclear Ub C RNA levels during all mutant virus infections, as

shown in fig 5.12.b, reflecting an increased relative rate of transcription. The increased level of RNA was only demonstrated in the cytoplasm of cells infected with a virus containing a functional E1A and E1B unit.

The rate of ubiquitin transcription was investigated in serum-deprived cells, infected with adenovirus mutants, by nuclear run on experiments. While it was not possible to differentiate between ubiquitin transcripts, there was a clear increase of the rate of transcription of ubiquitin in infected cells when compared to uninfected cells, as depicted in fig. 5.13. WT infection appeared to be the most efficient at inducing ubiquitin transcription, although viruses lacking functional E1A products (12S, 13S, E1Apm928 and hr3), E1B products (dl 313, dl 338, dl 337) and E4 products (dl 808) were able to increase the rate of transcription of ubiquitin, compared to the uninfected control. The anomaly in this experiment was the higher than expected induction of ubiquitin transcription by 12Spm961 infection. The 12Spm961 virus contains a mutation within the CD2 region within the 243aa protein. The expected induction would resemble the induction by E1Apm928 infection, as this virus also contains a mutation within CD2. Repeat experiments need to be performed to judge the validity of these results. The obvious trend, however, is that adenovirus infection stimulates the rate of ubiquitin transcription by at least two fold in the absence of serum.

Thus it appears that in the presence of serum, adenovirus infection does not increase the overall transcription rate of ubiquitin, as judged by nuclear RNA levels. Adenovirus infection results in the selective increase of Ub B RNA levels, as opposed to Ub C RNA levels in the cytoplasm, which reflects the constitutively increased Ub B transcription rate in the nucleus. The increase of Ub B mRNA levels in the cytoplasm is dependent on the expression of functional E1A and E1B products. In the absence of serum, adenovirus infection increases the overall rate of ubiquitin transcription.

Figure 5.12 Induction of Ubiquitin mRNA during mutant virus infection

a. 20µg total cytoplasmic RNA was isolated from uninfected cells and cells infected with WT, dl 312, 12S, 13S, dl 313, dl 327, dl 808 40 hrs pi, transferred to nylon membranes and probed with ubiquitin cDNA.

i. Northern analysis

ii. Densitometric comparison

- Ub C cytoplasmic mRNA levels
- Ub B cytoplasmic mRNA levels

b. 20µg total nuclear RNA was isolated from the same cells as above

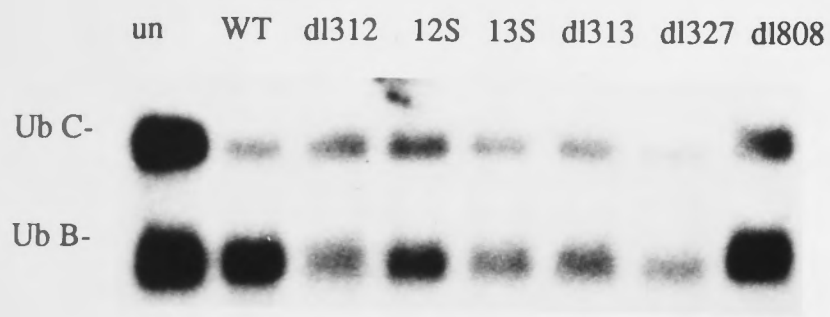
i Northern analysis

ii Densitometric comparison

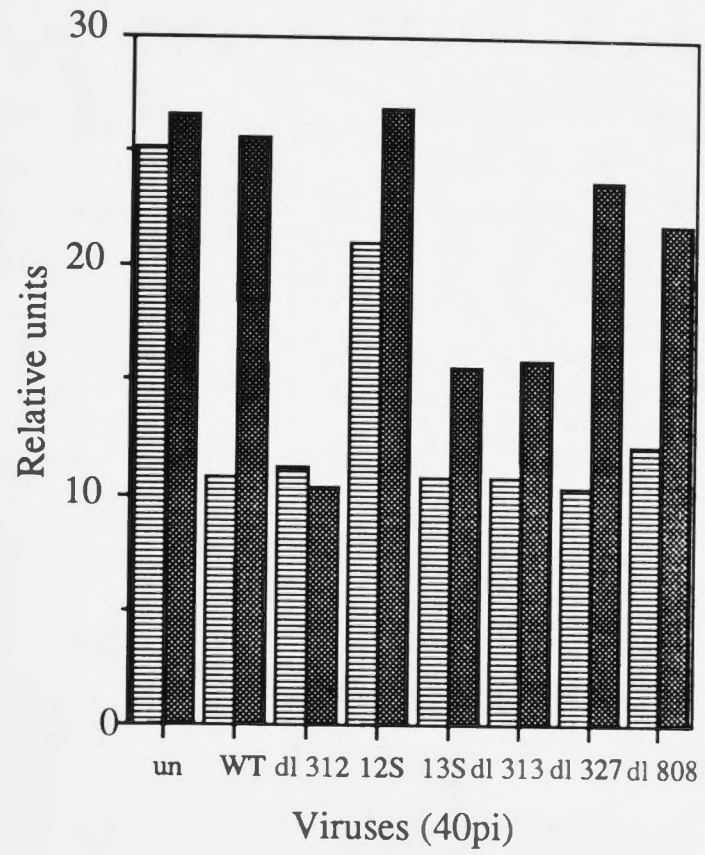
- Ub C nuclear mRNA
- Ub B nuclear mRNA

a. Induction of cytoplasmic Ubiquitin mRNA by mutant viruses

i. Northern analysis

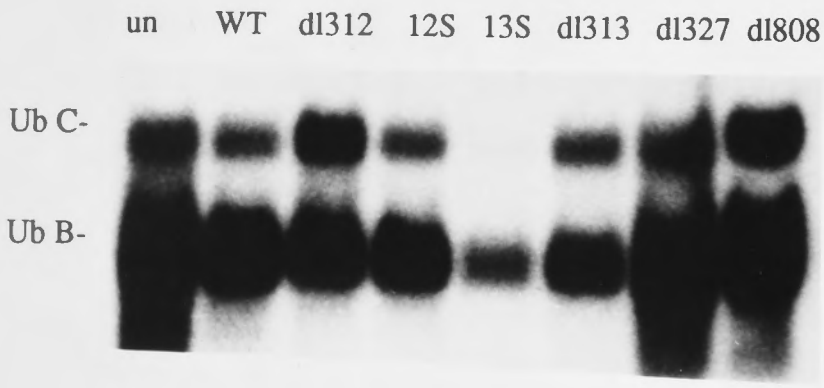


ii Relative comparison.

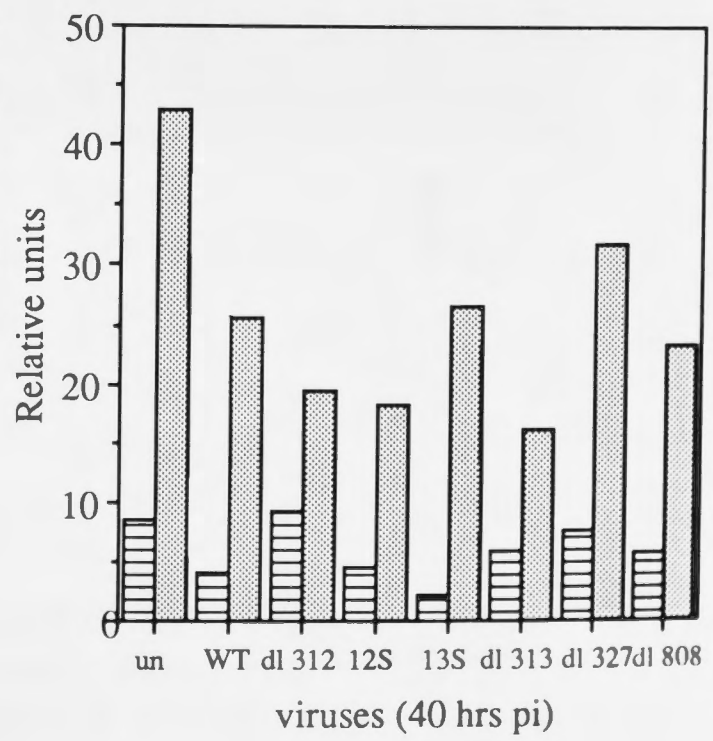


b. Induction of nuclear Ubiquitin mRNA by mutant viruses

i. Northern analysis



ii Relative comparison.



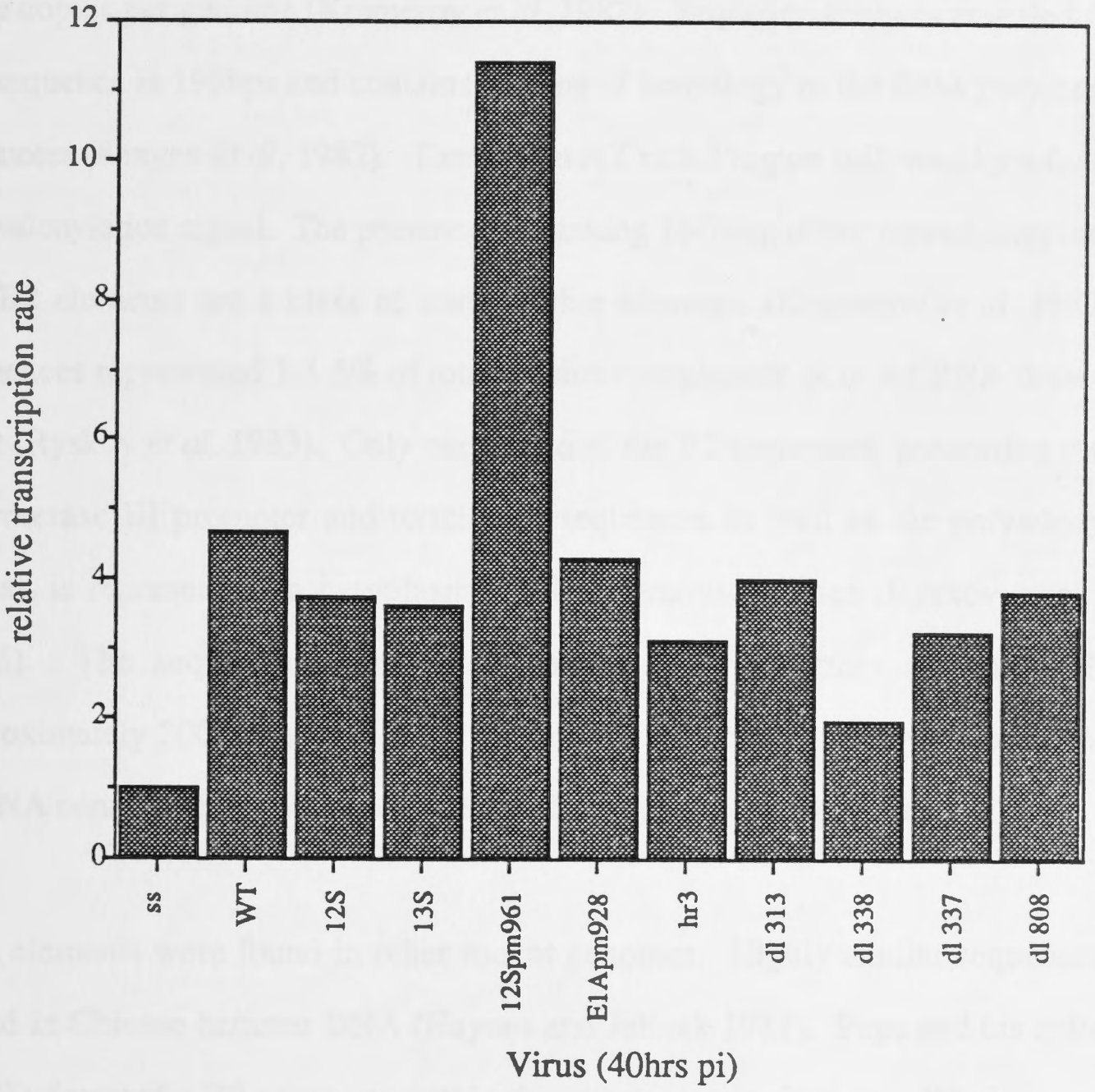


Figure 5.13 Transcription rate of ubiquitin during Ad mutant infection. Nuclei were isolated from serum deprived cells infected with WT, 12S, 13S, 12Spm961, E1Apm928, hr3, dl 313, dl 338, dl 337, dl808 40 hrs pi. Nascent RNA chains were elongated by RNA polymerase in the presence of a radiolabelled ribonucleotide, and used to probe ubiquitin cDNA immobilized on a nylon membrane.

▩ Relative ubiquitin transcription rate

Discussion

B2 repetitive sequences

B2 repetitive sequences were initially described by Kramerov and his colleagues (1979), as a class of abundant sequences of heterogeneous nuclear RNA (hnRNA) in the murine genome, that were able to form double-stranded structures after melting. Consequently, this class of repeat sequences were estimated at approximately $5x10^4$ gene copies per genome (Kramerov *et al.* 1982). Sequence analysis revealed that the B2 sequence is 190bps and contains regions of homology to the RNA polymerase III promoter (Krayev *et al.* 1982). There is an AT rich 3'region followed by a functional polyadenylation signal. The presence of flanking 15-16bp direct repeats suggested that the B2 elements are a class of transposable elements (Kramerov *et al.* 1982). B2 sequences represented 1-1.5% of total murine cytoplasmic poly A^+ RNA from mouse liver (Ryskov *et al.* 1983). Only one strand of the B2 sequences, possessing the RNA polymerase III promoter and terminator sequences as well as the polyadenylation signal, is represented in cytoplasmic RNA in mouse tissues (Ryskov *et al.* 1983, 1985). The sequences were found in three forms, either as small RNA of approximately 200-300bps, at the 3'terminus of high molecular weight mRNA, or as hnRNA constituents of RNA polymerase II transcripts (Ryskov *et al.* 1983).

B2 elements were found in other rodent genomes. Highly similar sequences were found in Chinese hamster DNA (Haynes and Jelinek 1981). Page and his colleagues (1981) detected a B2 sequence within the rat somatotropin gene. B2 elements were also found predominantly at the 3' terminus of a wide variety of genes, including genes for rat intestinal fatty acid binding protein, rat androgen binding protein, mouse β-glucuronidase, mouse RNA polymerase II large subunit, mouse urokinase-type plasminogen activator protein, mouse skeletal muscle phosphorylase kinase and mouse MHC class 1 H2-D (Sweetser *et al.* 1987, Joseph *et al.* 1988, D'Amore *et al.* 1987, Corden *et al.* 1985, Degen *et al.* 1988, Bender and Emerson 1987, Kress *et al.* 1984).

Although B2 sequences are found within other genes and transcribed as part of the genes by RNA polymerase II, small B2 RNA are actively synthesised by RNA polymerase III and not produced as a side product of processing hnRNA (Lekakh *et al.* 1984, Kramerov *et al.* 1985, Singh *et al.* 1985a).

B2 elements may identify mRNAs that are activated in oncogenesis and embryogenesis, although their role is uncertain. Murphy and his colleagues (1983) showed that the level of B2 RNA sharply increased at the 9-10 day stage of mouse embryogenesis, and that the level of B2 sequences in cytoplasmic poly A^+ RNA from undifferentiated embryonal carcinoma cells decreased upon differentiation. Enhanced levels of cytoplasmic B2 RNA were detected after stimulation of quiescent mouse fibroblasts by serum (Edwards *et al.* 1985). The transcription levels of B2 RNA were increased in a wide variety of murine transformed cell lines and tumours, compared to normal tissues (Ryskov *et al.* 1985, Grigoryan *et al.* 1985). SV40 transformed murine cell lines contained enhanced levels of B2 transcripts compared to the untransformed parental cell line (Singh *et al.* 1985a). B2 sequences were also found to be more abundantly expressed in a number of rat transformed cell lines (Kohnoe *et al.* 1987). The induction of B2 sequences in a variety of transformed cell lines implies that the increased levels of B2 elements may function in the maintenance of the transformed state. Perhaps the transcription and/or retrotransposition of these repetitive sequences may affect the expression and function of essential regulatory genes, as suggested by Grigoryan and his colleagues (1985). The small B2 RNA have been found in association with some mRNA in cytoplasmic ribonucleoproteins and associated with the cytoskeleton (Kramerov *et al.* 1985).

The present study shows that transcription of B2 repetitive sequences is induced during adenovirus infection. Two lines of evidence support this conclusion. Firstly, a cDNA clone (TD20), significantly similar to B2 sequences, was isolated by differential

screening of a subtracted library. The library, enriched for cellular sequences induced by E1A during adenovirus infection, was screened differentially using cDNA probes from E1A$^+$ and E1A$^-$ -infected cells, thus TD20 was likely to represent E1A-induced B2 sequences. Southern analysis of TD20 within uninfected rat DNA showed intense smearing indicative of a repetitive sequence.

The second more direct line of evidence that B2 sequences are induced during adenovirus infection stems from Northern analysis. A heterogeneous group of B2/TD20 transcripts, ranging in size from 150 to 400bps, increase in abundance 40 hrs after WT adenovirus infection. E1A alone was not responsible for the induction, as infection with mutants containing a viable E1A unit, but lacking functional E1B, E3 or E4 units failed to induce the transcription of the B2 sequences, implying a novel transactivating function (directly or indirectly) for E3 and E4 products. Alternatively, perhaps the induction of TD20 transcription was dependent on expression from the major late genes. Transcription from the major late promoter (MLP) occurs after the commencement of viral DNA synthesis, which is delayed during mutant virus infection.

Investigation of the rate of transcription of B2/TD20 revealed that infection with WT, E1A$^-$ and E1B$^-$ mutants increased the rate of transcription above the uninfected control. Viruses that contained mutations within the E1A putative CD3 domain had decreased efficiency of transactivation, and all viruses were not as efficient as WT. Interestingly, the reduced rate of transcription during E3 and E4 mutant infection, compared to uninfected control, once again implies that functional E3 and E4 proteins are necessary for transactivation of TD20.

While the transcription rate of TD20 increased during infection with WT, E1A$^-$ and E1B$^-$ mutants compared to uninfected controls, this increased rate led to a

corresponding increase in the level of TD20 cytoplasmic RNA only in WT infected cells, suggesting that functional viral early gene products are required to aid in increasing mRNA stability or transport from the nucleus to the cytoplasm. Such functions have already been assigned to the E1B and E4 proteins (as discussed in chapter 1).

It is not surprising that adenovirus infection induces the expression of B2 sequences. As mentioned above, it is well documented that B2 sequences are transcribed by RNA polymerase III. A number of investigators have shown that the E1A products enhance RNA polymerase III transcription. Gaynor and his colleagues (1985) showed an increase of transcription of transfected tRNA genes, viral VA-I and VA-II genes during infection. These genes are transcribed by RNA polymerase III. The transcription of endogenous cellular genes (tRNA and 5S RNA) was not stimulated. Nuclear extracts of infected cells isolated early in infection were found to have an increased capacity for RNA polymerase III transcription of exogenous VA RNA genes, 5S RNA and tRNA genes (Hoeffler and Roeder 1985). This increased capacity was due to the increased activity of the limiting cellular transcription factor IIIC (TFIIIC) (Yoshinaga *et al.* 1986). The studies to date only show increased transcription of exogenous genes by RNA polymerase III. The present study provides direct evidence that adenovirus infection stimulates the transcription of an endogenous gene transcribed by RNA polymerase III.

After isolation of B2 sequences by differential screening of a library made from heat shocked chinese hamster cells, B2 RNA levels were also shown to increase after heat shock and DNA damaging agents (Fornace and Mitchell 1986, Fornace *et al.* 1989a). Other members of heat shock responsive genes, eg. ubiquitin, were isolated from the library and found to be coordinately induced by heat shock (Fornace *et al.* 1989b). The effect of adenovirus infection on the expression of ubiquitin was further investigated.

Ubiquitin expression during adenovirus infection

Ubiquitin is a 76 amino acid protein that is conserved in all eukaryotic cells, implying a fundamental role in cell function. While ubiquitin is located as a monomer in cells, it is also conjugated to many proteins including histones 2A and 2B, and plasma membrane proteins, eg. lymphocyte homing receptor and the receptor for platelet-derived growth factor (Goldknopf and Busch 1977, West and Bonner 1980, Yarden *et al.* 1986, Siegelman *et al.* 1986). It has also been reported that ubiquitin has intrinsic proteolytic activity (Fried *et al.* 1987). One of the more important roles of ubiquitin is as a component in the ATP-dependent nonlysosomal protein degradation pathway, responsible for the degradation of damaged, denatured or short-lived proteins. This pathway is important in the heat shock response of eukaryotic cells. Ubiquitin is coordinately induced as a major mRNA transcript with the other major heat shock protein transcripts in heat stressed chicken embryo fibroblasts, chinese hamster cells and yeast cells (Bond and Schlesinger 1985, Fornace *et al.* 1989a,b, Finley *et al.* 1987). Adenovirus infection induces the transcription of heat shock protein genes and the induction is dependent on E1A expression (Kao and Nevins 1983, Wu *et al.* 1986, Simon *et al.* 1987). The close relationship between the induction of heat shock proteins and ubiquitin during stress warranted investigation of the effect of adenovirus infection on the expression of ubiquitin.

Three transcripts of ubiquitin are detected in rodent cells, similar to those detected in yeast, chicken and human (Fornace *et al.* 1989b, Taccioli *et al.* 1989, Bond and Schlesinger 1985, Baker and Board 1987). The two major transcripts of approximately 2.5kb and 1kb are transcribed from two genes, Ub C and Ub B respectively. These genes code for variable tandem repeats of the 76aa ubiquitin monomer. The third 0.6kb mRNA is transcribed from a ubiquitin fusion gene, Ub A. The Ub A genes code for a single ubiquitin monomer fused to either 52 or 76-80 amino acid peptides, which have recently been identified as members of a group of ribosomal

proteins. The transient association between ubiquitin and the ribosomal proteins is necessary for their incorporation into nascent ribosomes (Finley *et al.* 1989, Redman and Rechsteiner 1989). Others have shown that transcription from Ub A is not induced by heat shock or other stress (Finley *et al.* 1989, Taccioli *et al.* 1989).

Adenovirus infection induces the level of Ub B RNA within the cytoplasm. To investigate whether the transcription rate of Ub B was increased compared to Ub C, the levels of Ub B and Ub C within nuclear RNA were characterised. The alternative experiment to gain information about transcription rate (nuclear run on experiments) would not distinguish between the different ubiquitin genes. The results suggest that Ub B is constitutively transcribed at a higher rate than Ub C. In uninfected cells, the levels of Ub B and Ub C mRNA were equivalent at the cytoplasmic level, however, during adenovirus infection Ub B mRNA levels increased over Ub C levels in the cytoplasm. The greatest difference between cytoplasmic Ub B to Ub C levels occurred during WT infection, decreasing slightly with mutants lacking a functional E3 and E4 region and decreasing more significantly with E1A and E1B mutants. Thus, the increase of levels of cytoplasmic Ub B mRNA relative to Ub C levels relied on functional E1A and E1B transcription units. This may be another example of E1A products inducing transcription of E1B products, which act to specifically increase the stability or transport of the Ub B transcript. Thus, the results suggest that while there may be an increase in transcription of ubiquitin overall during adenovirus infection in serum-deprived cells, adenovirus does not elevate the transcription rate of the individual ubiquitin genes during infection of REFs in the presence of serum. Instead, the transport or stability of ubiquitin mRNA is selectively affected, leading to an increase of Ub B mRNA levels, and/or a reduction of Ub C mRNA levels in the cytoplasm.

The selective induction of cytoplasmic Ub B levels during herpes simplex virus (HSV) infection, dependent on the expression of the immediate early viral gene ICP4, has been shown by Latchman and his colleagues (1987). Functional ICP4 protein is essential for the transactivation of viral genes and certain cellular genes (Personn *et al.* 1985, Patel *et al.* 1986). Evidence was presented that the transcription rate of ubiquitin was increased during HSV infection, however it was not possible to discriminate between the transcription rates of Ub B and Ub C (Latchman *et al.* 1987). It is not known whether the induction of cytoplasmic ubiquitin mRNA levels by HSV is mediated through the induction of common regulatory transcription elements or changes in RNA transport or stability.

As mentioned above, ubiquitin is induced as a consequence of heat shock (Bond and Schlesinger 1985, Fornace *et al.* 1989a,b, Finley *et al.* 1987). The upstream region of a number of polyubiquitin genes contain the consensus heat shock element sequence (Ozkaynak *et al.* 1987, Bond and Schlesinger 1986, Swindle *et al.* 1988). Adenovirus induces the major HSP70 mRNA, at the level of transcription, mediated by the E1A gene products (Nevins 1982, Kao and Nevins 1983, Wu *et al.* 1986, Simon *et al.* 1987). On closer examination of the HSP70 promoter sequence requirements for E1A-mediated transactivation, it was found that a basal transcription complex of elements that interact with CCAAT transcription factor, SP1, TFIID and ATF/AP1-like factors was necessary for E1A transactivation (Williams *et al.* 1989). Mutations within any transcription element sequence did not abolish E1A-inducibility, but reduced E1A-induced levels and the basal transcription levels. The heat shock element was not necessary for E1A-transactivation. Thus the induction of HSP by E1A products and heat stress is mediated by different transcription complexes. The lack of E1A-inducibility through the heat shock element, lends support to the suggestion that E1A does not induce ubiquitin transcription. However, it should be noted that the presence

of other common transcription elements within the ubiquitin promoter has not been investigated.

Summary

The effect of adenovirus infection on the expression of two cellular sequences, B2 and ubiquitin, was investigated. B2 sequences were isolated from a subtractive cDNA library, enriched for E1A-induced cellular sequences. The level of cytoplasmic B2 RNA increased during adenovirus infection, due to an enhanced transcription rate. It is well documented that B2 sequences are transcribed by RNA polymerase III, therefore this study presented evidence that adenovirus induces RNA polymerase III transcription of an endogenous gene. Functional E1A, E1B, E3, and E4 regions were required for efficient transactivation and transport (or stabilisation) of B2 mRNA from the cytoplasm to the nucleus. Perhaps induction of B2 sequences is dependent on MLP expression, which begins after expression of all early genes and after viral DNA synthesis. The function of the B2 sequences is unknown, but the appearance of increased levels of B2 sequences is a common feature in transformed cells lines and during embryogenesis, implying an important role in cell growth.

The transcription rate of ubiquitin was unaffected by adenovirus infection of REFs in the presence of serum. There was a differential appearance of the two major ubiquitin transcripts in the cytoplasm during infection, which could be due to the selective stabilisation or transport of one of the transcripts, mediated by the E1A and E1B products. The experiments need to be repeated to ensure that the difference in expression is mediated by adenovirus infection and not merely by exposure to unique environmental stresses during an experiment. This differential expression of one of the ubiquitin gene products has been previously described during HSV infection. This phenomena, in infected semi-permissive cells, may result in an increase in the activity of the ubiquitin degradation pathway, leading to increased degradation of foreign viral

structural proteins and thus, an aborted lytic cycle. Perhaps this would aid the establishment of a transformed state.

Chapter 6

Discussion and conclusions

Discussion

The technique of subtractive hybridisation was used in the construction of a subtracted cDNA library and subsequent differential screening, to isolate cellular genes induced by E1A products during adenovirus infection of semi-permissive primary rodent cells. A small proportion of cells survive the encounter with the virus and become transformed, thus the putative cellular transcripts induced during infection, and isolated through the differential screening may be important in the establishment of a transformed phenotype.

Transformation does not occur in the absence of products from the early E1 region of adenovirus. The E1 region contains two transcription units, E1A and E1B. Rat cells transformed by the E1A region alone possess an immortalised phenotype (Houweling *et al.* 1980, Ruley 1983). The expression of E1B without E1A is not sufficient for transformation. Transformation of primary cells with E1A and E1B result in an oncogenic phenotype, characterised by anchorage-independent cell growth and the ability to form tumours in syngeneic animals.

The E1A products are able to activate the transcription of other viral early genes and have been documented to trans-activate transcription of certain cellular genes, eg. heat shock protein 70 (Kao and Nevins 1983). E1A gene products repress enhancer-mediated transcription from cellular genes, eg. the immunoglobulin heavy chain during infection of plasmocytoma cells (Hen *et al.* 1985), and viral genes, eg. repression of transcription from the SV40 early promoter in transient expression assays (Velcich and Ziff 1985). The E1A polypeptides also have mitogenic activity, inducing cellular DNA synthesis and mitosis (Bellett *et al.* 1985, Kaczmarek *et al.* 1986a, Zerler *et al.* 1987, Bellett *et al.* 1989). Due to the ability of E1A products to influence the expression of a variety of cellular gene products, including regulatory proteins, E1A products are central to the process of oncogenic transformation by adenoviruses.

Other investigators have isolated cellular sequences influenced by E1A through subtractive hybridisation. Hara and coworkers (1988) isolated several cDNA clones of cellular genes whose expression was repressed by adenovirus type 12 E1A gene products. They prepared a cDNA library from a rat 3Y1 (rat embryo fibroblast) cell line and differentially screened the library with labelled cDNA probes prepared from 3Y1 cells and a derivative cell line expressing E1A proteins constitutively. The expression of clones which preferentially hybridised to the cDNA probe from the 3Y1 cells was analysed during cell-cycle progression from G0 to G1 to S phase and in the presence of growth factors. Two clones were found to decrease transcriptionally in expression, after the addition of growth factors and/or the induction of the E1A gene. One of the clones was identified through sequence analysis as a fibronectin transcript, the level of which is lowered in E1A transformed cell lines (Roberts *et al.* 1985). Thus, E1A-induced transformation may be the result of a culmination of many complex cellular interactions, including the induction of expression of certain genes and the reduction of expression of others.

To isolate cellular transcripts induced in transformed cells, Kao and Nevins (1986) constructed a cDNA library from poly A^+ RNA from 293 cells (adenovirus-transformed human embryo kidney cells, Graham *et al.* 1977). The library was screened with a radiolabelled cDNA probe that was prepared from 293 poly A^+ RNA, hybridised to a large excess of RNA from a human embryonic kidney (HEK) cell line and passaged twice through hydroxylapatite. A number of cDNA sequences were isolated that represented mRNAs expressed at a higher level in 293 cells than in HEK cells. However, in consideration of the long history of 293 cells and the distant relationship between 293 cells and HEK cells, it is possible that the elevated sequences may be unrelated to the original E1A-induced transformation events and may be the result of an extreme phenotype.

The aim of the present study was to isolate cellular genes whose expression is induced by the E1A products during the early stages of infection, and may be important in transformation progression. cDNA was synthesised from poly A^+ RNA from G1-arrested REFS 40 hours after infection with WT, and hybridised with a 10 fold excess of poly A^+ RNA isolated from cells 40 hrs after infection with a mutant virus which lacks the E1A region (dl 312). Included in the hybridisation was an excess of adenoviral DNA. Single-stranded cDNAs, presumably representing unique cellular sequences induced by E1A products, were isolated after passage through a hydroxylapatite column and cloned into pUC 9, before transformation of bacteria, resulting in a subtracted cDNA library, enriched for E1A-induced cellular sequences.

After screening of the library with cDNA probes constructed from either WT infected REFs (the positive probe) or dl 312 infected REFs (the negative probe), clones were chosen that hybridised strongly to the positive probe, and negligibly to the negative probe. These clones were probed with adenoviral DNA, and did not hybridise to the probe, suggesting that any adenovirus cDNA sequences had hybridised to the excess of adenoviral DNA during the construction of the subtracted library and were removed by binding to the hydroxylapatite column.

The selected clones were also screened with the thymidine kinase gene, and did not show any homology to this gene. Thymidine kinase activity and mRNA levels in rodent cells increase approximately 3-4 fold after Ad5 infection. This increase is E1A dependent (Cheetham and Bellett 1982, Braithwaite *et al.* 1983). The lack of sequences homologous to the thymidine kinase gene among the induced clones suggests that the technique of subtractive cDNA cloning is not sensitive to minor inductions of transcripts. The cDNA from WT infected cells, used to make the library, was hybridised to a 10 fold excess of poly A^+ RNA from dl 312 infected cells, which

constitutively express a basal level of thymidine kinase. Thus it is likely that the slight induction of thymidine kinase cDNA was masked by the excess of thymidine kinase mRNA present at the hybridisation stage of library construction. It is possible that any cDNA representing constitutively expressed cellular transcripts tightly regulated in the absence of E1A, but slightly induced in the presence of E1A, eg. ß-tubulin (Stein and Ziff 1984), will be absent from the subtracted library, due to hybridisation to homologous mRNA present in excess, and removal by binding to the hydroxylapatite column used in library construction.

A proportion of differentially screened clones were characterised by restriction enzyme analysis, Northern analysis and sequencing. The inserts of eighteen cDNA clones ranged from 1kb to 0.13kb. The small sizes of the inserts may be a function of degradation during synthesis of the cDNA and during subtractive hybridisation. An alternative possibility is that the ligations were biased towards small insert fragments due to the higher molar ratio of small inserts and the lack of size fractionation of the cDNA before ligation. Plasmids with small inserts also have a greater transforming ability. Eighteen clones were subject to Northern analysis. Six of eighteen clones hybridised to multiple species of poly A^+ RNA from WT infected cells and not to poly A^+ RNA from dl 312 infected cells. Homology to adeno-associated virus (AAV) was detected through sequence analysis of four of the six cDNA inserts.

The adeno-associated viruses are members of the family Parvoviridae, and are dependent on coinfection with a helper virus (adenovirus, vaccinia, herpes simplex virus or human cytomegalovirus) for productive infection (Atchison *et al.* 1965, Rose and Koczot 1972, Schlehofer *et al.* 1986, Buller *et al.* 1981, McPherson *et al.* 1985). In the absence of a helper virus, AAV penetrate to the cell nucleus, where the DNA is uncoated and integrated efficiently into the genome. AAV can exist as a provirus for many cellular generations until rescue with a helper virus (Hoggan *et al.* 1972, Berns *et*

al. 1975, Handa *et al.* 1977). AAV was present in the adenovirus stocks used to infect the cells that were used in the cDNA library construction. It is believed that the contamination originated from some latently infected cell lines used to propagate viral stocks.

The presence of AAV during adenovirus infection of REFs complicates the investigation of cellular genes induced by E1A during infection. Co-infection of AAV with adenovirus results in a marked inhibition of adenovirus replication (Carter *et al.* 1979). The mechanism for this inhibition is unknown, although it can be overcome by infection with higher multiplicities of adenovirus, or preinfection of cells with adenovirus before infection with AAV (Hoggan *et al.* 1966, Parkes *et al.* 1968). This suggests a competition for cellular factors involved in DNA synthesis.

AAV can also inhibit adenovirus transformation and oncogenicity. AAV reduced the level of transformation of hamster embryo cells by adenovirus type 12 in tissue culture and reduced the oncogenicity of adenovirus-transformed hamster cell lines (Casto and Goodheart 1972, Ostrove *et al.* 1981). AAV coinfection inhibited adenovirus tumourigenesis in neonatal hamsters by 50% (de la Maza and Carter 1981). Adenovirus oncogenicity was inhibited by AAV DI particles and sheared infectious viral DNA, suggesting that the termini of AAV DNA are involved in inhibition (Cukor *et al.* 1984). Although the mechanism of AAV inhibition of viral transformation is not known, it may involve negative regulation of adenoviral gene expression. The AAV *rep* gene products are known to inhibit a variety of enhancer/promoter elements during stable and transient DNA plasmid transfections (Labow *et al.* 1986, 1987, Tratschin *et al.* 1986, Trempe and Carter 1988b). The inhibition of transformation by AAV is not absolute, thus the library was rescreened for cellular sequences induced during adenovirus infection in the presence or absence of AAV.

AAV sequences were identified in 71% of the clones that showed differential hybridisation in the original differential screenings. The remaining clones were rescreened with cDNA probes from WT infected cells and dl 312 infected cells. A clone was identified as cellular, and through Northern analysis, induced at the mRNA level during adenovirus infection in the presence or absence of AAV. After sequencing the cDNA insert, it was identified as a member of the B2 repetitive sequences (75% homology to the consensus sequence). This class of repeat sequences is estimated at approximately $5x10^4$ gene copies per genome (Kramerov *et al.* 1982). The consensus B2 sequence is 190bps and contains regions of homology to the RNA polymerase III promoter (Krayev *et al.* 1982). There is an AT rich 3'region followed by a functional polyadenylation signal. The presence of flanking 15-16bp direct repeats suggested that the B2 elements are a class of transposable elements (Kramerov *et al.* 1982).

The sequences are found in three forms, either as small RNA of approximately 200-300bps, at the 3' terminus of high molecular weight mRNA, or as hnRNA constituents of RNA polymerase II transcripts (Ryskov *et al.* 1983). Although B2 sequences are found within other genes and transcribed as part of the genes by RNA polymerase II, small B2 RNA are actively synthesised by RNA polymerase III and not produced as a side product of processing hnRNA (Lekakh *et al.* 1984, Kramerov *et al.* 1985, Singh *et al.* 1985a).

The function of B2 sequences is unknown. The transcription levels of B2 RNA were increased in a wide variety of murine transformed cell lines and tumours, compared to normal tissues (Ryskov *et al.* 1985, Grigoryan *et al.* 1985). SV40 transformed murine cell lines contained enhanced levels of B2 transcripts compared to the untransformed parental cell line (Singh *et al.* 1985a). B2 sequences were more abundantly expressed in a number of rat transformed cell lines (Kohnoe *et al.* 1987). The induction of B2 sequences in a variety of transformed cell lines implies that the increased levels of B2

elements may function in the maintenance of the transformed state. The transcription and/or retrotransposition of these repetitive sequences may affect the expression and function of essential regulatory genes, leading to deregulation of the cell cycle (Grigoryan *et al.* 1985). After isolation of B2 sequences by differential screening of a library made from heat shocked chinese hamster cells, B2 RNA levels were also shown to increase after heat shock and DNA damaging agents (Fornace and Mitchell 1986, Fornace *et al.* 1989a).

Adenovirus infection increases the expression of B2-related sequences at 30 to 40hrs pi. This may not be an indication of transformation progression, but may reflect a general increase of the expression of genes transcribed by RNA III polymerase. A number of investigators have shown that the E1A products enhance RNA polymerase III transcription (Gaynor *et.al.* 1985, Hoeffler and Roeder 1985). This increased transcription is due to the increased activity of the limiting cellular transcription factor IIIC (TFIIIC) (Yoshinaga *et al.* 1986). The studies to date only show increased transcription of exogenous transfected genes by RNA polymerase III. The present study provides direct evidence that adenovirus infection stimulates the transcription of an endogenous gene transcribed by RNA polymerase III.

Thus, the identification of a cDNA clone as a B2-related sequence, provides evidence that the library is enriched for E1A-induced sequences, as E1A induces RNA polymerase III activity, and B2 sequences are transcribed by RNA polymerase III. Similarly, the presence of AAV sequences amongst the cDNA clones indicates that the library was enriched for E1A-induced sequences, as dl 312 infection does not support AAV replication and transcription in REFs.

The isolation of a B2 repetitive sequence from the differentially screened clones, implies that the conditions of the subtractive hybridisation during library construction,

were not optimal for the isolation of rare transcripts from single copy genes. The hybridisation conditions of cDNA (WT infected cells) : mRNA (dl312 infected cells) : viral DNA during library construction, were established to titrate out all common cellular and viral sequences. The cDNA was hybridised to excess mRNA at a C_0t of 2,000 mol s l^{-1} (product of initial RNA concentration (molarity of nucleotides) and annealing time) to anneal repetitive cellular and viral sequences and leave unique sequences single stranded. It is possible that the repetitive sequence eluded the stringent hybridisation conditions, or binding to the hydroxylapatite column and was chosen, after differential screening, as an induced clone.

There is evidence, however, that the differential screening of the library was not entirely efficient. When a proportion of differentially screened clones were initially characterised by Northern analysis and sequencing, six of eighteen clones initially investigated hybridised to multiple species of poly A^+ RNA from WT infected cells and not to poly A^+ RNA from dl 312 and were identified as AAV sequences. The other 12 clones did not differentially hybridise to poly A^+ RNA from the WT infected cells. A member of the "non-induced" clones was sequenced and was significantly similar to a portion of the *CBP6* gene, which is a yeast nuclear gene necessary for the synthesis of mitochondrial cytochrome b (Dieckmann and Tzagoloff 1985). The clone was shown to be cellular. A mammalian equivalent of the yeast nuclear protein has not been identified. The sequence of the clone was similar to a small distinct region of the yeast gene, therefore the homology may reflect a conserved structural or regulatory domain, rather than a possible contamination of yeast in the cells used to make the library. The predicted secondary structure of the translated region of conservation was an α-helix. From current literature, it is not known whether this region is an important regulatory or structural domain. It is unlikely that this clone represents a gene that is induced during adenovirus infection, as there was no increase of mRNA expression during infection. Other investigators have identified anomalous yeast or mitochondrial

sequences while characterising differentially screened clones from a cDNA library (S.M. Phillips, personal communication, Parfett *et al.* 1989). It has been demonstrated that transcripts from the mitochondrial genome are found in preparations of total cytoplasmic RNA and form 0.2-0.5% of the poly A^+ mRNA (Edwards and Denhardt 1985).

The expression of a number of cellular genes are known to increase dramatically during adenovirus infection. The expression of 70kDa heat shock protein hsp gene (hsp70) increased by 100-fold, but only 2-fold in the absence of E1A function (Kao and Nevins 1983). The representative cDNA for hsp70 was not among the differentially screened clones isolated from the enriched library. The most likely reason for the under-representation of hsp70 sequences is that the induction of hsp70 in HeLa cells during infection peaked at 7 hrs and decreased by 91% by 13 hrs. Although there is no current evidence on kinetics of induction in primary REFs during infection, at the time of construction of the library (40hrs pi), the induction of hsp70 may not be significant.

AAV transcription had reached maximum activity 40 hrs after co-infection with WT, reflected in the high proportion of AAV sequences in the subtracted cDNA library. Correspondingly, induced-cellular sequences would be in lower proportion and more difficult to detect unless all AAV sequences were removed. When AAV sequences were removed, and the clones rescreened with cDNA probes, a repetitive sequence was isolated. Rare transcripts were not isolated after rescreening because cDNA probes were not enriched for rare transcripts. Several investigators have found that specific cDNA clones were not detected in plasmid libraries if 0.05% to 0.2% of their probes did not contain the gene of interest (Dworkin and David 1980, Crampton *et al.* 1980). Rare transcripts (0.001-0.005% abundance) would be undetectable with unfractionated cDNA probes, thus the clones should be rescreened with enriched cDNA probes (Davis *et al.* 1984a).

While the present study did not identify and characterise cellular genes induced by E1A products that play crucial roles in the establishment of the immortalized phenotype, a number of E1A-induced sequences were identified, including AAV sequences indicating AAV contamination within adenovirus stocks, and B2-related sequences. B2 sequences have been previously isolated by differential screening of a library made from heat shocked chinese hamster cells (Fornace and Mitchell 1986). The effect of adenovirus infection on the expression of another heat shock responsive gene, ubiquitin, was further investigated.

Ubiquitin is a component in the ATP-dependent nonlysosomal protein degradation pathway, responsible for the degradation of damaged, denatured or short-lived proteins. The transcription rate of ubiquitin was unaffected by adenovirus infection. There was a differential appearance of the two major ubiquitin transcripts in the cytoplasm during infection, which could be due to the selective stabilisation or transport of one of the transcripts and may result in an increase in the activity of the ubiquitin degradation pathway, leading to increased degradation of foreign viral structural proteins and thus, an aborted lytic cycle. The activity of ubiquitin within the protein degradation pathway has yet to be monitored in infected cells to substantiate the hypothesis. The differential expression of one of the ubiquitin gene products has been previously described during HSV infection (Latchman *et al.* 1987).

Future Directions

Subtractive cDNA libraries have been used in many instances to isolate rare cellular transcripts that are present in one cellular population, but absent in a closely related cellular population that lacks the specific phenotype under investigation. The technique is valuable because it allows the isolation of genes that may influence the specific phenotype, without prejudice towards previously described genes. To minimise

irrelevant transcripts and to exploit the technique to its full potential, the original populations of cells must be closely related, and differ only in the phenotype in question.

In the present study, the cell populations were identical, differing only in the virus used to infect the cells. The presence of AAV introduced a further complication, therefore another subtracted library should be constructed with AAV free viruses and cells. To accurately represent cellular sequences induced directly by E1A gene products, the library should be constructed from RNA isolated at an earlier time during infection than 40hrs (which is after the start of viral DNA replication and expression of all viral genes). As E1A mRNA has been detected at 12-15hrs pi, perhaps the library should be constructed from cells infected for no more than 20hrs. Transcriptional repression is believed to play an important role in transformation (Lillie *et al.* 1986, Moran *et al.* 1986b). To isolate cellular sequences repressed by E1A products during infection, cDNA made from dl 312 infected cells should be hybridised with poly A^+ RNA from WT infected cells before passage through hydroxylapatite, ligation and transformation.

Once cDNA clones have been isolated by screening with subtracted probes, the clones would be characterised by cross-hybridisation studies, Northern analysis to determine the extent of induction, and sequencing. Analysis of induction of expression in other adenovirus transformed cell lines would indicated whether expression of the clones were necessary for maintenance of transformation. Analysis of expression in cells transformed by carcinogen treatment or other oncogenes could indicate whether the induction of clones is a general feature of transformation. A full length cDNA clone could be isolated from cDNA library constructed from poly A^+ RNA from infected REFS, and the protein identified from *in vitro* translation. Monoclonal antibodies could be raised against the protein, to study interactions with other proteins, by Western analysis and protein location, by immunofluorescence. To assign functions to

the E1A-influenced proteins, the proteins could be microinjected into the cell, or the full length cDNA could be placed in front of a strong promoter and transfected into REFs. Disruption to the cell-cycle progression would indicate a role for the cDNA. Alternatively, antisense oligonucleotides or RNA could be introduced during infected or into transformed cells, to functionally inactivate the specific RNA, and possibly affect the transformation efficiency of adenovirus. Antisense RNA and oligonucleotides have been used successfully to abrogate the function of specific mRNA *in vivo* (Green *et al.* 1986, Heikella *et al.* 1987, Jaskulski *et al.* 1988). These approaches to characterise the effect of a single gene product, are not sensitive to the full scope of complex cellular responses to E1A products, to create an intracellular environment ultimately resulting in immortality.

Clearly, future molecular and genetic analysis of the influence of E1A gene products on unknown genes isolated from cDNA libraries as discussed above, and previously described genes, isolated through their involvement in the regulation of cell cycle progression, will gradually lead to a better understanding of the processes involved in transformation.

Bibliography

Abate, C., Luk, D., Reiner, G., Rauscher III, F.J. and Curran, T. (1990). Expression and purification of the leucine zipper and DNA-binding domains of Fos and Jun: both Fos and Jun contact DNA directly. *Proc. Natl. Acad. Sci. USA* **87**:1032-1036.

Akusjarvi, G., Mathews, M.B., Andersson, P., Vennstrom, B. and Pettersson, U. (1980). Structure of genes for virus-associated RNAI and RNAII of adenovirus type 2. *Proc. Natl. Acad. Sci. USA* **77**:2424-2428.

Alestrom, P., Akusjarvi, G., Perricaudet, M., Mathews, M.B., Klessig, D.F. and Pettersson, V. (1980). The gene for polypepetide IX of adenovirus type 2 and its unspliced messenger RNA. *Cell* **19**:671-681.

Anderson, K.P. and Klessig, D.F. (1983). Posttranscriptional block to synthesis of a human adenovirus capsid protein in abortively infected monkey cells*J. Mol. Appl. Genet.* **2**:31-43.

Anderson, K.P. and Klessig, D.F. (1984). Altered mRNA splicing in monkey cells abortively infected with human adenovirus may be responsible for inefficient synthesis of the virion fiber polypeptide. *Proc. Natl. Acad. Sci. USA* **81**:4023-4027.

Andersson, M., Paabo, S., Nilsson, T. and Peterson, P.A. (1985). Impaired intracellular transport of class 1 MHC antigens as a possible means for adenoviruses to evade immune surveillance. *Cell* **43**:215-222.

Arrand, J.R. and Roberts, R.J. (1979). The nucleotide sequences at the termini of adenovirus-2 DNA. *J. Mol. Biol.* **128**:577-594.

Atchison, R.W., Casto, B.C. and Hammon, W.McD (1965). Adenovirus-associated defective virus particles. *Science* **149**:754-756.

Babich, A., Feldman, L.T., Nevins, J.R., Darnell Jr., J.E. and Weinberger, C. (1983). Effect of adenovirus on metabolism of specific host mRNA: transport control and specific translational discrimination. *Mol. Cell. Biol.* **3**:1212-1221.

Babich, A. and Nevins, J.R. (1981). The stability of early adenovirus mRNA is controlled by the viral 72kd DNA binding protein. *Cell* **26**:371-379.

Babiss, L.E. (1989). The cellular transcription factor E2f requires viral E1A and E4 gene products for increased DNA-binding activity and functions to stimulate adenovirus E2A gene expression. *J. Virol.* **63**:2709-2717.

Babiss, L.E. and Ginsberg, H.S. (1984). Adenovirus type 5 early region 1B gene product is required for efficient shutoff of host protein synthesis. *J. Virol.* **50**:202-212.

Baker, R.T. and Board, P.G. (1987). The human ubiquitin gene family: structure of a gene and pseudogenes from the Ub B subfamily. *Nucl. Acids Res.* **15**:443-463.

Baker, S.J., Fearon, E.R., Nigro, J.M., Hamilton, S.R., Preisinger, A.C., Jessup, J.M., van Tuinen, P., Ledbetter, D.H., Barter, D.F., Nakamura, Y., White, R. and Vogelstein, B. (1989). Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* **244**:217-221.

Barker, D.D. and Berk, A.J. (1987). Adenovirus proteins from both E1B reading frames are required for transformation of rodent cells by viral infection and DNA transfection. *Virology* **156**:107-121.

Bedard, P.-A., Alcorta, D., Simmons, D.L., Luk, K.-C. and Erikson, R.L. (1987). Constitutive expression of a gene encoding a polypeptide homologous to biologically active human platelet protein in Rous sarcoma virus-tranformed fibroblasts. *Proc. Natl. Acad. Sci. USA* **84**:6715-6719.

Beladi, I. Adenoviruses. In: *Strains of Human Viruses*, edited by Majer, M. and Plotkin, S.A. (1972), Karger, Basel, p.1.

Bellett, A.J.D., Jackson, P., David, E., Bennett, E.J. and Cronin, B. (1989). Functions of the two adenovirus early E1A protens and their conserved domains in cell-cycle alteration, actin reorganisation and gene activation in rat cells. *J. Virol.* **63**:303-310.

Bellett, A.J.D., Li, P., David, E.T., Mackey, E.J., Braithwaite, A.W. and Cutt, J.R. (1985). Control functions of adenovirus transformation region E1A gene products in rat and human cells. *Mol. Cell. Biol.* **5**:1933-1939.

Bello, L.T. and Ginsberg, H.S. (1967). Inhibition of host protein synthesis in type 5 adenovirus-infected cells. *J. Virol.* 1:843-850.

Beltz, G.A. and Flint, S.J. (1979). Inhibition of HeLa cell protein synthesis during adenovirus infection. *J. Mol. Biol.* **131**:353-373.

Bender, P.K. and Emerson Jr., C.P. (1987). Skeletal muscle phosphorylase kinase catalytic subunit mRNAs are expressed in heart tissue but not in liver. *J. Biol. Chem.* **262**:8799-8805.

Berget, S., Moore, C. and Sharp, P.A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc. Natl. Acad. Sci. USA* **74**:3171-3175.

Berk, A.J., Lee, F., Harrison, T., Williams, J. and Sharp, P.A. (1979). Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs. *Cell* **17**:935-944.

Berk, A.J. and Sharp, P.A. (1977). Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. *Cell* **12**:721-732.

Bernards, R., de Leeuw, M.G., Houweling, A. and van der Eb, A.J. (1986). Role of the adenovirus early region 1B tumor antigens in tranformation and lytic infection. *Virology* **150**:126-139.

Berns, K.I., Kort, J., Fife, K.H., Grogon, E.W. and Spear, I. (1975). Study of the fine structure of adeno-associated virus DNA with bacterial restriction endonucleases. *J. Virol.* **16**:712-719.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucl. Acids Res.* **7**:1513-1523.

Birrer, M.J., Segal, S., DeGreve, J.S., Kaye, F., Sausville, E.A. and Minna, J.D. (1988). L-*myc* co-operates with *ras* to transform primary rat embryo fibroblasts. *Mol. Cell. Biol.* **8**:2668-2673.

Blair-Zajdel, M.E. and Blair, G.E. (1988). The intracellular distribution of the transformation-associated protein p53 in adenovirus-transformed rodent cells. *Oncogene* **2**:579-584.

Bond, U. and Schlesinger, M.J. (1986). The chicken ubiquitin gene contains a heat shock promoter and expresses an unstable mRNA in heat-shocked cells. *Mol. Cell. Biol.* **6**:4602-4610.

Bond, U. and Schlesinger, M.J. (1985). Ubiquitin is a heat shock protein in chicken embryo fibroblasts. *Mol. Cell. Biol.* **5**:949-956.

Borelli, E., Hen, R. and Chambon, P. (1984). Adenovirus-2 E1A products repress enhancer-induced stimulation of transcription. *Nature* **312**:608-612.

Bos, J.L., Polder, L.J., Bernards, R., Schrier, P.I., van der Elsen, P.J., van der Eb, A.J. and van Ormondt, H. (1981). The 22kb E1B mRNA of human Ad 12 and Ad5 codes for two tumour antigens starting at different AUG triplets. *Cell* **27**:121-131.

Brady, H.A. and Wold, W.S.M. (1988). Competition between splicing and polyadenylation determines which adenovirus region E3 mRNAs are synthesised. *Mol. Cell. Biol.* **8**:3291-3297.

Braithwaite, A., Nelson, C., Skulimowski, A., McGovern, J., Pigott, D. and Jenkins, J. (1990). Transactivation of the p53 oncogene by E1A gene products. *Virol.* (submitted)

Braithwaite, A.W., Cheetham, B.F., Li, P., Parish, C.R., Waldron-Stevens, L.K. and Bellett, A.J.D. (1983). Adenovirus-induced alternations of the cell growth cycle: a requirement for expression of E1A but not E1B. *J. Virol.* **45**:192-199.

Braithwaite, A.W., Murray, J.D. and Bellett, A.J.D. (1981). Alterations to controls of cellular DNA synthesis by adenovirus infection. *J. Virol.* **39**:331-340.

Bravo, R., Frank, R., Blundell, P.A. and Macdonald-Bravo, H. (1987). Cyclin/PCNA is the auxiliary protein of DNA polymerase δ. *Nature* **326**:575-517.

Bridge, E. and Ketner, G. (1989). Redundant control of adenovirus late gene expression by early region 4. *J. Virol.* **63**:631-638.

Buchkovich, K., Duffy, L. and Harlow, E. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. *Cell* **58**:1097-1105.

Buller, R.M., Janik, J.E., Sebring, E.D. and Rose, J.A. (1981). Herpes simplex virus types 1 and 2 completely help adenovirus-associated virus replication. *J. Virol.* **40**:241-247.

Burdon, R.H. (1986). Heat shock and the heat shock proteins. *Biochem.J.* **240**:313-324.

Burgert, H.G. and Kvist, S. (1985). An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class 1 antigens. *Cell* **41**:987-997.

Calabretta, B., Kaczmarek, L., Mars, W., Ochoa, D., Gibson, C.W., Hirschhorn, R.R. and Baserga, R. (1985). Cell-cycle-specific genes differentially expressed in human leukemias. *Proc. Natl. Acad. Sci. USA* **52**:4463-4467.

Carlin, C.R., Tollefson, A.E., Brady, H.A., Hoffman, B.L. and Wold, W.S.M. (1989). Epidermal growth factor receptor is down-regulated by a 10,400 mw protein encoded by the E3 region of adenovirus. *Cell* **57**:135-144.

Carter, B.J., Laughlin, C.A., de la Maza, L.M. and Myers, M. (1979). Adeno-associated virus autointerference. *Virology* **92**:449-462.

Carter, T.H. and Blanton, R.A. (1978). Possible role of the 72,000 dalton DNA-binding protein in regulation of adenovirus type 5 early gene expression. *J. Virol.* **25**:664-674.

Castiglia, C.L. and Flint, S.J. (1983). Effects of adenovirus infection on rRNA synthesis and maturation in HeLa cells. *Mol. Cell. Biol.* **3**:662-671.

Casto, B.C. and Goodheart, C.R. (1972). Inhibition of adenovirus transformation *in vitro* by AAV-1. *Proc. Soc. Exp. Biol. Med.***140**:72-78.

Ceredig, R., Medveczky, J. and Skulimowski, A. (1989). Mouse fetal thymus lobes cultured in IL-2 generate $CD3^+$, TCR-γδ-expressing $CD4^-/CD8^+$ and $CD4^-/CD8^-$ cells. *J. Immunol.***142**:3353-3360.

Challberg, S.S. and Ketner, G. (1981). Deletion mutants of adenovirus 2: Isolation and initial characterization of virus carrying mutations near the right end of the viral genome. *Virology* **114**:196-209.

Chatterjee, P.K., Bruner, M., Flint, S.J. and Harter, M.L. (1988). DNA-binding properties of an adenovirus 289R E1A protein. *The EMBO J.* **7**:835-841.

Cheetham, B.F. and Bellett, A.J.D. (1982). A biochemical investigation of the adenovirus-induced G1 to S phase progression: thymidine kinase, ornithine decarboxylase, and inhibitors of polyamine biosynthesis. *J. Cell. Physiol.* **110**:114-122.

Cheung, A.K.M., Hoggan, M.D., Hauswirth, W.W. and Berns, K.I. (1980). Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. *J. Virol.* **33**:739-748.

Chinnadurai, G. (1983). Adenovirus Ip^+ locus codes for a 19kd tumor antigen that plays an essential role in cell transformation. *Cell* **33**:759-766.

Chirico, W.J., Waters, M.G. and Blobel, G. (1988). 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* **332**:805-810.

Chiu, R., Boyle, W.J., Meek, J., Smeal, T., Hunter, T. and Karin, M. (1988). The c-*fos* protein interacts with c*Jun*/AP-1 to stimulate transcription of AP-1 responsive genes. *Cell* **54**:541-552.

Chou, P.Y. and Fasman, G.D. (1978). Prediction of the secondary structure of proteins from their amino acid sequence. *Adv. Enzymol.* **47**:45-148.

Chow, L.T. and Broker, T.R. (1978). The spliced structures of adenovirus 2 fiber message and the other late mRNAs. *Cell* **15**:497-510

Chow, K.-C. and Pearson, G.D. (1985). Adenovirus infection elevates levels of cellular topoisomerase 1. *Proc. Natl. Acad. Sci. USA* **82**:2247-2251.

Chow, L.T., Broker, T. and Lewis, J.B. (1979). The complex splicing patterns of RNA from the early regions of Ad 2. *J. Mol. Biol.* **134**:265-303.

Chow, L.T., Gelinas, R.E., Broker, T.R. and Roberts, R.J. (1977). An amazing sequence arrangement at the 5' ends of adenovirus 2 messenger RNA. *Cell* **12**:1-8.

Ciechanover, A., Finley, D. and Varshavsky, A. (1984). The ubiquitin-mediated proteolytic pathway and mechanisms of energy-dependent intracellular protein degradation. *J. Cell. Biochem.* **24**:27-53.

Cladaras, C. and Wold, W.S.M. (1985). DNA sequence of the early E3 transcription unit of adenovirus 5. *Virology* **140**:28-43.

Cleghan, V.G. and Klessig, D.F. (1986). Association of the adenovirus DNA-binding protein with RNA both *in vitro* and *in vivo*. *Proc. Natl. Acad. Sci. USA* **83**:8947-8951.

Cochran, B.H., Reffel, A.C. and Stiles, C.D. (1983). Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell* **33**:939-947.

Cochran, B.H., Zullo, J., Verma, I.M. and Stiles, C.D. (1984). Expression of the c*fos* gene and of a *fos*-related gene is stimulated by platelet-derived growth factor. *Science* **226**:1080-1082.

Corden, L.J., Cadena, D.L., Ahearn, J.M. and Dahmus, M.E. (1985). A unique structure at the carboxyl terminus of the largest subunit of eukaryotic RNA polymerase II. *Proc. Natl. Acad. Sci. USA* **82**:7934-7938.

Crampton J., Humphries, S., Woods, D., and Williamson, R. (1980). The isolation of clones cDNA sequences which are differentially expressed in human lymphocytes and fibroblasts. *Nucl. Acids Res.* **8**:6007-6017.

Crawford, L.V., Pim, D.C., Gurney, E.G., Goodfellow, P. and Taylor-Papadimitriou, J. (1981). Detection of a common feature in several human tumor cell lines - a 53,000-dalton protein. *Proc. Natl. Acad. Sci. USA* **78**:41-45.

Cukor, G., Blacklow, N.R., Hoggan, M.D. and Berns, K.I. (1984). Biology of adeno-associated virus. In *The Parvoviruses*, edited by Berns, K.I. (1984), New York: Plenum, pp 33-66.

Culp, J.S., Webster, L.C., Friedman, D.J., Smith, C.L., Huang, W-J., Wu, F.Y-H., Rosenberg, M. and Ricciardi, R.P. (1988). The 289-amino acid E1A protein of adenovirus binds zinc in a region that is important for trans-activation. *Proc. Natl. Acad. Sci. USA* **85**:6450-6454.

Cutt, J.R., Shenk, T. and Hearing, P. (1987). Analysis of adenovirus early reigion 4-encoded polypeptides synthesized in productively infected cells. *J. Virol.* **61**:543-552.

D'Amore, M.A., Gallagher, P.M., Korfhagen, T.R. and Ganschow, R.E. (1988). The complete sequence and organization of the murine ß-glucuronidase gene. *Biochemistry* **27**:7131-7140.

Davis, M.M., Chien Y.-H., Gascoigne, N.R.J. and Hedrick, S.M. (1984a). A murine T cell receptor gene complex: isolation, structure and rearrangement. *Immunol. Rev.* **81**:235-258.

Davis, M.M., Cohen, D.I., Nielson, E.A., Steinmetz, M., Paul, W.E. and Hood, L. (1984b). Cell-type-specific cDNA probes and the murine I region: the localization and orientation of Adα. *Proc. Natl. Acad. Sci. USA* **81**:2194-2198.

De Caprio, J.A., Ludlow, J.W., Figge, J., Shew, J-Y., Huang, C-M., Lee, W-H., Marsilio, E., Paucha, E. and Livingston, D.M. (1988). SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. *Cell* **54**:275-283.

De Fromentel, C.C., Viron, A., Puvion, E. and May, P. (1986). SV40 large T-antigen and transformation related protein p53 are associated *in situ* with nuclear RNP structures containing hnRNA of transformed cells. *Exp. Cell. Res.* **164**:35-48.

Dear, T.N., Ramshaw, I.A. and Kefford, R.F. (1988). Differential expression of a novel gene WDNM1 in a nonmetastatic rat mammary adenocarcinoma cells. *Cancer Res.* **48**:5203-5209.

Degen, S.J.F., Heckel, J.L., Reich, E. and Degen, J.L. (1988). The murine urokinase-type plasminogen activator gene. *Biochemistry* **26**:8270-8279.

DeLeo, A.B., Jay, G., Appella, E., Dubois, G.C., Law, L.W. and Old, L.J. (1979). Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. USA* **76**:2420-2424.

Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. and Schekman, R. (1988). A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides. *Nature* **332**:800-805.

De la Maza, L.M. and Carter, B.J. (1980a). Molecular structure of adeno-associated virus variant DNA. *J. Biol. Chem.* **255**:3194-3202.

De la Maza, L.M. and Carter, B.J. (1980b). Heavy and light particles of adeno-associated virus. *J. Virol.* **33**:1129-1137.

De la Maza, L.M. and Carter, B.J. (1981). Inhibition of adenovirus oncogenicity by adeno-associated virus DNA. *J. Natl. Cancer Inst.* **67**:1323-1326.

Dieckmann, C.L. and Tzagoloff, A. (1985). Assembly of the mitochondrial membrane system *CBP6*, a yeast nuclear gene necessary for synthesis of cytochrome b. *J. Biol. Chem.* **260**:1513-1520.

Distal, R.J., Ro, H.-S., Rosen, B.S., Groves, D.L. and Spiegelman, B.M. (1987). Nucleoprotein complexes that regulate gene expression in adipocyte differentiation: direct participation of c*fos*. *Cell* **49**:835-844.

Doerfler, W. and Lundholm, V. (1970). Absence of replication of the DNA of adenovirus type 12 in BHK 21 cells. *Virology* **40**:754-757.

Draetta, G. and Beach, D. (1988). Activation of cdc2 protein kinase during mitosis in human cells. Cell cycle-dependent phosphorylation and subunit rearrangement. *Cell* **54**:17-26.

Draetta, G., Beach, D. and Moran, E. (1988). Synthesis of p34, the mammalian homolog of the yeast cdc2$^+$/cdc28 protein kinase, is stimulated during adenovirus-induced proliferation of primary baby rat kidney cells. *Oncogene* **2**:553-557.

Draetta, G., Brizuela, L., Potashkin, J. and Beach, D. (1987). Identification of p34 and p13, human homologs of the cell cycle regulators of fission yeast encoded by cdc2$^+$ and suc1$^+$. *Cell* **50**:319-325.

Duggleby, R.G., Kinns, H. and Rood, J.J. (1981). A computer program for determining the size of DNA restriction fragments. *Anal. Biochem.* **110**:49-55.

Dunphy, W.G., Brizuela, L., Beach, D. and Newport, J. (1988). The Xenopus cdc2 protein is a component of MPF, a cytoplasmic regulator of mitosis. *Cell* **54**:423-431.

Dworkin, M.B. and David, I.B. (1980). Construction of a cloned library of expressed embryonic gene sequences from *Xenopus laevis*. *Develop. Biol.* **76**:435-448.

Dyson, N., Buchkovich, K., Whyte, P. and Harlow, E. (1989a). The cellular 107K protein that binds to adenovirus E1A also associates with the large T antigens of SV40 and J.C. virus. *Cell* **58**:249-255.

Dyson, N., Howley, P.M., Munger, K. and Harlow, E. (1989b). The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. *Science* **243**:934-937.

Edwards, D.R. and Denhardt, D.T. (1985). A study of mitochondrial and nuclear transcription with cloned cDNA probes. *Exp. Cell Res.* **157**:127-143.

Edwards, D.R., Parfett, C.L.J. and Denhardt, D.T. (1985). Transcriptional regulation of two serum-induced RNAs in mouse fibroblasts: Equivalence of one species to B2 repetitive elements. *Mol. Cell. Biol.* **5**:3280-3288.

Egan, C., Bayley, S.T. and Branton, P.E. (1989). Binding of the Rb1 protein to E1A products is required for adenovirus transformation. *Oncogene* **4**:383-388.

Eggerding, F.A. and Pierce, W.C. (1986). Molecular biology of adenovirus type 2 semipermissive infections 1. viral growth and expression of viral replicative functions during restricted adenovirus infection. *Virology* **148**:97-113.

Eliyahu, D., Goldfinger, N., Pinhasi-Kimhi, O., Shaulsky, G., Skurnik, Y., Arai, N., Rotter, V. and Oren, M. (1988). Meth A fibrosarcoma cells express two transforming mutant p53 species. *Oncogene* **3**:313-321.

Eliyahu, D., Michalovitz, D., Eliyahu, S., Pinhasi-Kinhi, O. and Oren, M. (1989). Wild-type p53 can inhibit oncogene-mediated focus formation. *Proc. Natl. Acad. Sci. USA* **86**:8763-8767.

Eliyahu, D., Raz, A., Gruss, P., Givol, D. and Oren, M. (1984). Participation of p53 cellular antigen in transformation of normal embryonic cells. *Nature* **312**:646-649.

Engel, D.A., Hardy, S. and Shenk, T. (1988). cAMP acts in synergy with E1A protein to activate transcription of the adenovirus early genes E4, and E1A. *Genes Devel.***2**:1517-1528.

Fahnestock, M.L. and Lewis, J.B. (1989). Genetic dissection of the transactivating domain of the E1A 289R protein of adenovirus type 2. *J. Virol.* **63**:1495-1504.

Falgout, B.N. and Ketner, G. (1987). Early region 4 is required for the efficient assembly of adenovirus particles. *J. Virol.* **67**:3759-3768.

Ferguson, B., Krippl, B., Andrisani, O., Jones, N. and Westphal, H. (1985). E1A 13S and 12S mRNA products made in *Escherichia coli* both function as nucleus-localized transcription activators but do not directly bind DNA. *Mol. Cell. Biol.* **5**:2653-2661.

Finlay, C.A., Hinds, P.W. and Levine, A.J. (1989). The p53 proto-oncogene can act as a suppressor of transformation. *Cell* **57**:1083-1093.

Finlay, C.A., Hinds, P.W., Tan, T-H., Eliyahu, D., Oren, M. and Levine, A.J. (1988). Activating mutations for transformation by p53 produced a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol. Cell. Biol.* **8**:531-539.

Finley, D., Bartel, B. and Varshavski, A. (1989). The tails of ubiquitin precursors are ribosomal proteins whose fusion to ubiquitin facilitates ribosome biogenesis. *Nature* **338**:394-401.

Finley, D., Ozkaynak, E. and Varshavsky, (1987). The yeast polyubiquitin gene is essential for resistance to high temperatures, starvation and other stresses. *Cell* **48**:1035-1046.

Fornace Jr., A.J., Alamo Jr., I., Hollander, M.C. and Lamoreaux, E. (1989a). Induction of heat shock protein transcripts and B2 transcripts by various stresses in chinese hamster cells. *Exp. Cell. Res.* **182**:61-74.

Fornace Jr., A.J., Alamo Jr., I., Hollander, M.C. and Lamoreaux, E. (1989b). Ubiquitin mRNA is a major stress induced transcript in mammalian cells. *Nucl. Acids Res.* **17**:1215-1230.

Fornace Jr, A.J. and Mitchell, J.B. (1986). Induction of B2 RNA polymerase III transcription by heat shock: enrichment for heat shock induced sequences in rodent cells by hybridization subtraction. *Nucl. Acids Res.* **14**:5793-5811.

Francoeur, A.M. and Mathews, M.B. (1982). Interaction between VA RNA and the lupus antigen La: formation of a ribonucleoprotein *in vitro*. *Proc. Natl. Acad. Sci. USA* **79**:6772-6776.

Fraser, N. and Ziff, E. (1978). RNA structures near poly (A) of adenovirus-2 late messenger RNAs. *J. Mol. Biol.* **124**:27-51.

Fraser, N.W., Nevins, J.R., Ziff, E. and Darnell Jr., J.E. (1979). The major late adenovirus type 2 transcription unit: termination is downstream from the last poly (A) site. *J. Mol. Biol.* **129**:643-656.

Fried, V.A., Smith, H.T., Hildebrandt, E. and Weiner, K. (1987). Ubiquitin has intrinsic proteolytic activity: implications for cellular regulation. *Proc. Natl. Acad. Sci. USA* **84**:3685-3689.

Friefeld, B.R., Krevolin, M.D. and Horwitz, M.S. (1983). Effects of the adenovirus H5ts125 and H5ts107 DNA binding proteins on DNA replication *in vitro*. *Virology* **124**:380-389.

Friend, S.H., Bernard, R., Rogelj, S., Weinberg, R.A., Rapaport, J.M., Albert, D.M. and Dryja, T.P. (1986). A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* **323**:643-646.

Fryer, G.A., Katoh, Y. and Roberts, R.J. (1984). Characterization of the major mRNAs from adenovirus 2 early region 4 by cDNA cloning and sequencing. *Nucl. Acids Res.* **12**:3503-3519.

Gallimore, P.H., Byrd, P.J. and Grand, R.J.A. Adenovirus genes involved in transformation. What determines the oncogenic phenotype?. In: *Viruses and Cancer*, edited by Rigby P.W.J. and Wilkie N.M. (1985), Cambridge University Press. Cambridge pp125-172.

Gallimore, P.H., Sharp, P.A. and Sambrook, J. (1974). Viral DNA in transformed cells: A study of the sequences of adenovirus 2 DNA in nine lines of transformed rat cells using specific fragments of the viral genome. *J. Mol. Biol.* **89**:49-72.

Galos, R.S., Williams, J., Burger, M.H. and Flint, S.J. (1979). Localization of additional early gene sequences in the adenoviral chromosome. *Cell* **17**:945-956.

Gambke, C. and Deppert, W. (1983). Specific complex of the late nonstructural 100,000-dalton protein with newly synthesised hexon in adenovirus type 2-infected cells. *Virology* **124**:1-12.

Garon, C.F., Berry, K.W. and Rose, J.A. (1972). A unique form of terminal redundancy in adenovirus DNA molecules. *Proc. Natl. Acad. Sci. USA* **69**:2391-2395.

Garon, C.F., Parr, R.P., Padmanabhan, R., Roninson, I., Garrison, J.W. and Rose, J.A. (1982). Structural characterisation of the adenovirus 18 inverted terminal repetition. *Virology* **121**:230-239.

Gaynor, R.B., Feldman, L.T. and Berk, A.J. (1985). Transcription of class III genes activated by viral immediate early genes. *Science* **230**:447-450.

Gaynor, R.B., Hillman, D. and Berk, A.J. (1984). Adenovirus early region 1A activates transcription of a nonviral gene introduced into mammalian cells by infection or transfection. *Proc. Natl. Acad. Sci. USA* **81**:1193-1194.

Gibbs, A.J. and McIntyre, G.A. (1970). The diagram, a method for comparing sequences. *Eur. J. Biochem.***16**:1-11.

Gibson, C.W., Rittling, S.R., Hirschhorn, R.R., Kaczmarek, L., Calabretta, B., Stiles, C.D. and Baserga, R. (1986). Cell cycle dependent genes induced by different mitogens in cells from different species. *Mol. Cell. Biol.* **71**:61-69.

Ginsberg, H.S., Bello, L.J. and Levine, A.J. Control of biosynthesis of host macromolecules in cells infected with adenovirus. In: *The Molecular Biology of Viruses*, edited by Colter, J.S. and Paranchych, W. (1967), New York: Academic Press, p. 547.

Giordano, A., Whyte, P., Harlow, E., Franza Jr., B.R., Beach, D. and Draetta, G. (1989). A 60kd cdc2-associated polypepetide complexes with the E1A proteins in adenovirus-infected cells. *Cell* **58**:981-990.

Glenn, G.M. and Ricciardi, R.P. (1985). Adenovirus 5 early region 1A host range mutants hr3, hr4 and hr5 contain point mutations which generate single amino acid substitutions. *J. Virol.* **56**:66-74.

Godbout, R., Dryja, T.P., Squire, J., Gallie, B.L. and Philips, R.A. (1983). Somatic inactivation of genes on chromosome 13 is a common event in retinoblastoma. *Nature* **304**:451-453.

Goldberg, S., Nevins, J. and Darnell, J.E. (1978). Evidence from UV transcription mapping that late adenovirus type 2 mRNA is derived from a large precursor molecule. *J. Virol.* **25**:806-810.

Goldknopf, I.L. and Busch, H. (1977). Isopeptide linkage between non histone and histone 2A polypeptides of chromosomal conjugate-protein A24. *Proc. Natl. Acad. Sci. USA* **74**:864-868.

Gooding, L.R., Elmore, L.W., Tollefson, A.E., Brady, H.A. and Wold, W.S.M. (1988). A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell* **53**:341-346.

Gottlieb, J. and Muzyczka, N. (1988). *In vitro* excision of adeno-associated virus DNA from recombinant plasmids: isolation of an enzyme fraction from HeLa cells that cleaves DNA at poly (G) sequences. *Mol. Cell. Biol.* **8**:2513-2522.

Graham, F.L., Smiley, J., Russell, W.C. and Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59-72.

Graham, F.L., van der Eb, A.J. and Heijneker, H.L. (1974). Size and location of the transforming region in human adenovirus DNA. *Nature* **251**:687-691.

Grand, R.J.A., Byrd, P.S., Grabham, P.W., Gregory, C.D., Huen, D.S., Merrick, R.M., Young, L.S. and Gallimore, P.H. (1989). The expression of the retinoblastoma gene product Rb1 in primary and adenovirus-transformed human cells. *Oncogene* **4**:1291-1298.

Green, M., Loewenstein, P.M., Pusztai, R. and Symington, J.S. (1988). An adenovirus E1A protein domain activates transcription *in vivo* and *in vitro* in the absence of protein synthesis. *Cell* **53**:921-926.

Green, M., Mackey, J., Wold, W.S.M. and Rigden, P. (1979). Thirty-one human adenovirus serotypes (Ad1-Ad31) form five groups (A-E) based on DNA genome homologies. *Virology* **93**:481-492.

Green, M., Pina, M., Kimas, R., Wensink, P., MacHattie, L. and Thomas, C.A.I. (1967). Adenovirus DNA molecular weight and conformation. *Proc. Natl. Acad. Sci. USA* **57**:1302-1309.

Green, M.R. and Roeder, R.G. (1980). Transcripts of the adeno-associated virus genome: mapping of the major RNAs. *J. Virol.* **36**:79-92.

Green, M.R., Straus, S.E. and Roeder, R.G. (1980). Transcripts of the adeno-associated virus genome: multiple polyadenylated RNAs including a potential primary transcript. *J. Virol.* **35**:560-565.

Green, M.R., Treisman, R. and Maniatis, T. (1983). Transcriptional activation of cloned ß-globin genes by viral immediate-early gene products. *Cell* **35**:137-148.

Green, P.J., Pines, O. and Inouye, M. (1986). The role of antisense RNA in gene regulation. *Ann. Rev. Biochem.* **55**:569-597.

Greenberg, M.E. and Ziff, E.B. (1984). Stimulation of 3T3 cells induces transcription of the c-*fos* proto-oncogene. *Nature* **311**:433-439.

Grigoryan, M.S., Kramerov, D.A., Tulchinsky, E.M., Revasova, E.S. and Lukanidin, E.M. (1985). Activation of putative transposition intermediate formation in tumor cells. *The EMBO J.* **4**:2209-2215.

Halbert, D.N., Cutt, J.R. and Shenk, T. (1985). Adenovirus early region 4 encodes functions required for efficient DNA replication late gene expression and host cell shutoff. *J. Virol.* **56**:250-257.

Handa, H., Shiroki, K. and Shimojo, H. (1977). Establishment and characterization of KB cell lines latently infected with adeno-associated virus type 1. *Virology* **82**:84-92.

Hara, E., Nakada, S., Takehana, K., Nakajima, T., Iino,T. and Oda,K. (1988). Molecular cloning and characterization of cellular genes whose expression is repressed by the adenovirus E1A gene products and growth factors in quiescent rat cells. *Gene* **70**:97-106.

Harbour, J.W., Lai, S.-L., Whang-Peng, J., Gazdar, A.F., Minna, J.D. and Kaye, F.S. (1988). Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. *Science* **241**:253-257.

Harlow, E., Franza Jr., B.R. and Schley, C. (1985). Monoclonal antibodies specific for adenovirus early region 1A proteins: Extensive heterogeneity in early region 1A products. *J. Virol.* **55**:533-546.

Harlow, E., Whyte, P., Franza Jr., B.R. and Schley, C. (1986). Association of adenovirus early-region 1A proteins with cellular polypeptides. *Mol. Cell. Biol.* **6**:1579-1589.

Harrison, T., Graham, F. and Williams, J. (1977). Host-range mutants of adenovirus type 5 defective for growth in HeLa cells. *Virology* **77**:319-329.

Hartley, J.W. and Rowe, W.P. (1960). A new mouse virus apparently related to the adenovirus group. *Virology* **11**:645-647.

Hauswirth, W.W. and Berns, K.I. (1977). Origin and termination of adeno-associated virus DNA replication. *Virology* **78**:488-499.

Hauswirth, W.W. and Berns, K.J. (1979). Adeno-associated virus DNA replication: nonunit-length molecules. *Virology* **93**:57-68.

Haynes, S.R. and Jelinek, W.R. (1981). Low molecular weight RNAs transcribed *in vitro* by RNA polymerase III from Alu-type dispersed repeats in chinese hamster DNA are also found *in vivo*. *Proc. Natl. Acad. Sci. USA* **78**:6130-6134.

Hedrick, S.M., Cohen, D.I., Nielsen, E.A. and Davis, M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature* **308**:149-153.

Heikella, R., Schab, G., Wickstrom, E., Loong Loke, S., Pluznik, D.H., Watt, R. and Neckers, L.M. (1987). A c-*myc* antisense oligonucleotide inhibits entry into S phase but not progress from G0 toG1. *Nature* **328**:445-449.

Hen, R., Borrelli, E. and Chambon, P. (1985). Repression of the immunoglobulin heavy chain enhancer by the adenovirus-2 ElA products. *Science* **230**:1391-1394.

Herbst, R.S., Hermo Jr., H., Fisher, P.B. and Babiss, L.E. (1988). Regulation of adenovirus and cellular gene expression and of cellular transformation by the E1B-encoded 175 amino-acid protein. *J. Virol.* **62**:4634-4643.

Herisse, J., Rigolet, M., Du Pont de Dinechin, S. and Galibert, F. (1981). Nucleotide sequence of adenovirus 2 DNA fragment encoding for the carboxylic region of the fiber protein and the entire E4 region. *Nucl. Acids Res.* **9**:4023-4042.

Hicks, G.G. and Mowat, M. (1988). Integration of Friend murine leukemia virus into both alleles of p53 oncogene in an erythroleukemic cell line. *J. Virol.* **62**:4752-4755.

Hilleman, M.R. and Werner, J.H. (1954). Recovery of a new agent from patients with acute respiratory illness. *Proc. Soc. Exp. Biol. Med.* **85**:183-188.

Hind, R.W., Finlay, C.A. and Levine, A.J. (1989). Mutation is required to activate the p53 gene for cooperation with the *ras* oncogene and transformation. *J. Virol.* **63**:739-746.

Hinds, P.W., Finlay, C.A., Frey, A.B. and Levine, A.J. (1987). Immunological evidence for the association of p53 with a heat shock protein, hsc70 in p53-plus-*ras*-transformed cell lines. *Mol. Cell. Biol.* 7:2863-2869.

Hirschhorn, R.R., Aller, P., Zhi-An Yuan, Gibson, C.W. and Baserga, R. (1984). Cell-cycle specific cDNAs from mammalian cells temperature sensitive for growth. *Proc. Natl. Acad. Sci. USA* **81**:6004-6008.

Hirschhorn, R.R., Marashi, F., Baserga, R., Stein, J. and Stein, G. (1984). Expression of histone genes in a G1-specific temperature-sensitive mutant of the cell cycle. *Biochemistry* **23**:3731-3753.

Hodgson, C.P. and Fisk, R.Z. (1987). Hybridization probe size control: optimized oligolabelling. *Nucl. Acids Res.* **15**:6295.

Hoeffler, W.K., Kovelman, R. and Roeder, R.G. (1988). Activation of transcription factor IIIC by the adenovirus E1A protein. *Cell* **53**:907-920.

Hoeffler, W.K. and Roeder, R.G. (1985). Enhancement of RNA polymerase III transcription by the E1A gene product of adenovirus. *Cell* **41**:995-963.

Hoggan, M.D., Blacklow, N.R., and Rowe, W.P. (1966). Studies of small DNA viruses found in various adenovirus preparations: Physical, biological, and immunological characteristics. *Proc. Natl. Acad. Sci. U.S.A.* **55**:1467-1474.

Hoggan, M.D., Thomas, G.F. and Johnson, F.B. Continuous "carriage" of adenovirus-associated virus genome in cell culture in the absence of helper adenovirus. In *Possible Episomes in Eukaryotes Proceedings of the Fourth Lepetit Colloquium,* Cocoyoc, Mexico, edited by Silvestri, L.G.(1972), Amsterdam :North Holland Publishing Company, pp 243-249.

Horowitz, J.M., Yandell, D.W., Park, S.-H., Canning, S., Whyte, P., Buchkovich, K., Harlow, E., Weinberg, R.A. and Dryja, T.P. (1989). Point mutational inactivation of the retinoblastoma antioncogene. *Science* **243**:937-940.

Houweling, A., van der Elsen, P.J. and van der Eb, A.J. (1980). Partial Transformation of primary rat cells by the leftmost 4.5% fragment of adenovirus 5 DNA. *Virology* **105**:537-550.

Huang, H.-J.S., Yee, J.-K., Shew, J.-Y., Chen, P.-L., Bookskin, R., Freidmann, T., Lee, E.Y.-H.P. and Lee, W.-H. (1988). Suppression of the neoplastic phenotype byy replacement of the RB gene in human cancer cells. *Science* **242**:1563-1566.

Huebner, R.J., Casey, M.J., Chanock, R.M. and Schell, K. (1965). Tumours induced in hamsters by a strain of adenovirus type 3. Sharing of tumour antigens and "neoantigens" with those produced by adenovirus type 1 tumours. *Proc. Natl. Acad. Sci. USA* **54**:381-388.

Huebner, R.J., Rowe, W.P. and Lane, W.T. (1962). Oncogenic effects in hamsters of human adenovirus types 12 and 18. *Proc. Natl. Acad. Sci. USA* **48**:2051-2058.

Hull, R.N., Johnson, I.S., Culbertson, C.G., Reimer, C.B. and Wright, H.F. (1965). Oncogenicity of the simian adenoviruses. *Science* **150**:1044-1046.

Hull, R.N., Minner, J.R. and Mascoli, C.C. (1958). New viral agents recovered from tissue cultures of monkey cells. III additional agents from cultures of monkey tissues and directly from tissues and exreta. *Am. J. Hyg.* **68**:31.

Hurwitz, D.R. and Chinnadurai, G. (1985). Immortalization of rat embryo fibroblasts by an adenovirus 2 mutant expressing a single functional E1A protein. *J. Virol.* **54**:358-363.

Ish-Horowicz, D. and Burke, J.F. (1981). Rapid and efficient cosmid cloning. *Nucl. Acids Res.* 9:2989-2998.

Jackson, P. and Bellett, A.J.D. (1985). Reduced microfilament organization in adenovirus type 5-infected rat embryo cells: a function of early region 1A. *J.Virol.* **55**:644-650.

Jackson, P. and Bellett, A.J.D. (1987). The organization of vinculin and α-actinin in adenovirus 5-infected rat embryo cells. *Eur. J. Cell. Biol.***44**:161-165.

Jackson, P. and Bellett, A.J.D. (1989). Relationship between organization of the actin cytoskeleton and the cell cycle in normal and adenovirus-infected rat cells. *J. Virol.* **63**:311-318.

Janik, J.E., Huston, M.M., Cho, K. and Rose, J.A. (1989). Efficient synthesis of adeno-associated virus structural proteins requires both adenovirus DNA binding protein and VAI RNA. *Virology* **168**:320-329.

Janik, J.E., Huston, M.M. and Rose, J.A. (1981). Locations of adenovirus genes required for the replication of adeno-associated virus. *Proc. Natl. Acad. Sci. USA* **78**:1925-1929.

Jaskulski, D., de Riel, J.K., Mercer, E., Calbretta, B. and Baserger, R. (1988). Inhibition of cellular proliferation by antisense oligodeoxynucleotides to PCNA cyclin. *Science* **240**:1544-1546.

Jay, F.T., Laughlin, C.A. and Carter, B.J. (1981). Eukaryotic translational control: adeno-associated virus protein synthesis is affected by a mutation in the adenovirus DNA-binding protein. *Proc. Natl. Acad. Sci. USA* **78**:2927-2931.

Jelsma, T.N., Howe, J.A., Evelegh, C.M., Cunniff, N.F., Skiadopoulos, M.H., Floroft, M.R., Denman, J.E. and Bayley, S.T. (1988). Use of deletion and point mutants spanning the coding region of the adenovirus 5 E1A gene to define a domain that is essential for transcriptional activation. *Virology* **163**:494-502.

Jelsma, T.N., Howe, J.A., Mymryk, J.S., Evelegh, C.M., Cunniff, N.F.A. and Bayley, S.T. (1989). Sequences in E1A proteins of human adenovirus 5 required for cell transformation, repression of a transcriptional enhancer and induction of proliferating cell nuclear antigen. *Virology* **171**:120-130.

Jenkins, J.R., Rudge, K., Chumakor, P. and Currie, G.A. (1985). The cellular oncogene p53 can be activated by mutagenesis. *Nature* **317**:816-818.

Jenkins, J.R., Rudge, K. and Currie, G.A. (1984). Cellular immortalization by a cDNA clone encoding the transformation-associated phosphoprotein p53. *Nature* **312**:651-654.

Jochemsen, A.G., Peltenburg, L.T.C., Te Pas, M.F.W., de Wit, C.M., Bos, J.L. and van der Eb, A.J. (1987). Activation of adenovirus 5 E1A transcription by region E1B in transformed primary rat cells. *The EMBO J.* **6**:3399-3405.

Johnston, J.M., Anderson, K.P. and Klessig, D.F. (1985). Partial block to transcription of human adenovirus type 2 late genes in abortively injected monkey cells. *J. Virol.* **56**:378-385.

Jones, N. and Shenk, T. (1979a). An adenovirus early gene function regulates expression of other early viral genes. *Proc. Natl. Acad. Sci. USA* **76**:3665-3669.

Jones, N. and Shenk, T. (1979b). Isolation of adenovirus type 5 host range deletion mutants defective for transformation of rat embryo cells. *Cell* **17**:683-689.

Joseph, D.R., Hall, S.H., Conti, M. and French, F.S. (1988). The gene structure of rat androgen-binding protein: identification of potential regulatory deoxyribonucleic acid elements of a follicle-stimulating hormone-regulated protein. *Mol. Endocrin. Symp.* **2**:3-13.

Kabsch, W. and Sander, C. (1983). How good are predictions of protein secondary structure?. *FEBS Lett.***155**:179-182.

Kaczmarek, L., Ferguson, B., Rosenberg, M. and Baserga, R. (1986a). Induction of cellular DNA synthesis by purified adenovirus E1A proteins. *Virology* **152**:1-10.

Kaczmarek, L., Oren, M. and Baserga, R. (1986b). Co-operation between the p53 protein tumor antigen and platelet-poor plasma in the induction of cellular DNA synthesis. *Exp. Cell. Res.* **162**:268-272.

Kao, H.T., Capassa, O., Heintz, N. and Nevins, J.R. (1985). Cell cycle control of the human HSP70 gene: implications for the role of a cellular E1A-like function. *Mol. Cell. Biol.* **5**:628-633.

Kao, H.T. and Nevins, J.R. (1983). Transcriptional activation and subsequent control of the human heat shock gene during adenovirus infection. *Mol. Cell. Biol.* 3:2058-2065.

Kao, H.T. and Nevins, J.R. (1986). Alterations of cellular gene expression in adenovirus transformed cells by post-transcriptional mechanisms. *Nucl. Acids Res.* **14**:7253-7263.

Kapsenberg, J.G. (1959). Relationship of infectious canine hepatitis virus to human adenovirus. *Proc. Soc. Exp. Biol. Med.***101**:611-614.

Katze, M.G., de Corato, D., Safer, B., Galabru, J. and Hovanessian, A.G. (1987). Adenovirus VA1 RNA complexes with the 68,000 Mr Protein kinase to regulate its autophosphorylation and activity. *The EMBO J.* **6**:689-697.

Kelly, K., Cochran, B.H., Stiles, C.D. and Leder, P. (1983). Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factors. *Cell* **35**:603-610.

Kelly, T.J. and Lewis, A.M.J. (1973). Use of non-defective adenovirus-simian virus 40 hybrids for mapping the simian virus 40 genome. *J. Virol.* **12**:643-652.

Kibrick, S., Melendez, L. and Enders, J.F. (1957). Clinical associations of enteric viruses with particular reference to agents exhibiting properties of the ECHO group. *Ann. N.Y. Acad. Sci.* **67**:311-325.

Kitchingman, G.R., Lai, S.P. and Westphal, H. (1977). Loop structures in hybrids of early RNA and the separated strands of adenovirus DNA. *Proc. Natl. Acad. Sci. USA* **74**:4392-4395.

Kitchingman, G.R. and Westphal, H. (1980). The structure of adenovirus 2 early nuclear and cytoplasmic RNA. *J. Mol. Biol.* **137**:23-48.

Klessig, D.F. and Grodzicker, T. (1979). Mutations that allow human Ad 2 and Ad 5 to express late genes in monkey cells map in the viral gene encoding the 72K DNA binding protein. *Cell* **17**:957-966.

Klessig, D.F., Rice, S.A., Cleghon, V., Brough, D.E., Williams, J.F. and Voelkerding, K. Studies on the adenovirus DNA-binding protein. In: *Cancer Cells and DNA Tumor Viruses: Control of Gene expression and Replication*, (1986), New York: Cold Spring Harbor Laboratory, p. 485-496.

Koczot, F.J., Carter, B.J., Garon, C.F. and Rose, J.A. (1973). Self-complementarity of terminal sequences within plus or minus strands of adenovirus-associated virus DNA. *Proc. Natl. Acad. Sci. USA* **70**:215-219.

Kohnoe, S., Maehara, Y. and Endo, H. (1987). A systematic survey of repetitive sequences abundantly expressed in rat tumors. *Biochim. Biophys. Acta* **909**:107-114.

Kotin, R.M. and Berns, K.I. (1989). Organisation of adeno-associated virus DNA in latently infected Detroit 6 cells. *Virology* **170**:460-467.

Kovesdi, I., Reichel, R. and Nevins, J.R. (1986). Identification of a cellular transcription factor involved in ElA trans-activation. *Cell* **45**:219-228.

Kovesdi, I., Reichel, R. and Nevins, J.R. (1987). Role of an adenovirus E2 promoter binding factor in EIA-mediated coordinate gene control. *Proc. Natl. Acad. Sci. USA* **84**:2180-2184.

Kouzarides, T. and Ziff, E. (1988). The role of the leucine zipper in the fos-jun interaction. *Nature* **336**:646-651.

Kraiss, S., Quaiser, A., Oren, M. and Montenarh, M. (1988). Oligomerization of oncoprotein p53. *J. Virol.* **62**:4737-4744.

Kramerov, D.A., Grigoryan, A.A., Ryskov, A.P. and Georgiev, G.P. (1979). Long double-stranded sequences (ds RNA-B) of nuclear pre-mRNA consist of a few highly abundant classes of sequences: evidence from DNA cloning experiments. *Nucl. Acids Res.* **6**:697-713.

Kramerov, D.A., Lekakh, I.V., Samarina, O.P. and Ryskov, A.P. (1982). The sequences homologous to major interspersed repeats B1 and B2 of the mouse genome are present in mRNA and small cytoplasmic poly $(A)^+$ RNA. *Nucl. Acids Res.* **10**:7477-7491.

Kramerov, D.A., Tillib, S.V., Lekakh, I.V., Ryskov, A.P. and Georgiev, G.P. (1985). Biosynthesis and cytoplasmic distribution of small poly (A) containing B2 RNA. *Biochim. Biophys. Acta* **824**:85-98.

Krayev, A.S., Markusheva, T.V., Kramerov, D.A., Ryskov, A.P., Skryabin, K.G., Bayev, A.A. and Georgiev, G.P. (1982). Ubiquitous transposon-like repeats B1 and B2 of the mouse genome: B2 sequencing. *Nucl. Acids Res.* **23**:7461-7475.

Kress, M., Barra, Y., Seidman, J.G., Khoury, G. and Jay, G. (1984). Functional insertion of an Alu type 2 (B2 SINE) repetitive sequence in murine class 1 genes. *Science* **226**:974-977.

Kruijer, W., Cooper, J.A., Hunter, T. and Verma, I.M. (1984). Platelet-derived growth factor induces rapid but transient expression of the c*fos* gene and protein. *Nature* **312**:711-716.

Kuppuswamy, M.N. and Chinnadurai, G. (1987). Relationship between the transforming and transcriptional regulatory functions of adenovirus 2 E1A oncogene. *Virology* **159**:31-38.

Labow, M.A., Graf, L.H. and Berns, K.I. (1987). Adeno-associated virus gene expression inhibits cellular transformation by heterologous genes. *Mol. Cell. Biol.* **7**:1320-1325.

Labow, M.A., Hermonat, P.L. and Berns, K.I. (1986). Positive and negative autoregulation of the adeno-associated virus type 2 genome. *J. Virol.* **60**:251-258.

Land, H., Parada, L.F. and Weinberg, R.A. (1983). Tumorigenic conversion of primary embryo fibroblasts requires at least two co-operating oncogenes. *Nature* **304**:596-602.

Landschulz, W.H., Johnson, P.F. and McKnight, S.L. (1988). The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**:1759-1764.

Lane, D.P. and Crawford, L.V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**:261-263.

Latchman, D.S., Estridge, J.K. and Kemp, L.M. (1987). Transcriptional induction of the ubiquitin gene during herpes simplex virus infection is dependent upon the viral immediate-early protein ICP4. *Nucl. Acids Res.* **15**:7283-7293.

Laughlin, C.A., Jones, N. and Carter, B.J. (1982). Effect of deletions in adenovirus early region 1 genes upon replication of adeno-associated virus. *J. Virol.* **41**:868-876.

Laughlin, C.A., Westphal, H. and Carter, B.J. (1979). Spliced adeno-associated virus RNA. *Proc. Natl. Acad. Sci. USA* **76**:5567-5571.

Lawler, S.H., Jones, R.W., Eliceiri, B.P. and Eliceiri, G.L. (1989). Adenovirus infection retards ribosomal RNA processing. *J. Cell. Physiol.* **138**:205-207.

Ledinko, N. (1966). Changes in metabolic and enzymic activities of monkey kidney cells after infection with adenovirus 2. *Virology* **28**:679-692.

Ledinko, N. (1968). Enhanced deoxyribonucleic acid polymerase activity in human embryonic kidney cells infected with adenovirus 2 or 12. *J. Virol.* **2**:89-98.

Lee, E.Y.-H., To, H., Shew, J.Y., Bookstein, R., Scully, P. and Lee, W.-H. (1988). Inactivation of the retinoblastoma susceptibility gene in human breast cancers. *Science* **241**:218-221.

Lee, K.A.W., Hai, T.Y., Siva Raman, L., Thimmappaya, B., Hurst, H.C., Jones, N.C. and Green, M.R. (1987). A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters. *Proc. Natl. Acad. Sci. USA* **84**:8355-8359.

Lee, W-H., Bookstein, R., Hong, F., Young, L.H., Shew, J.Y. and Lee, E.Y.-H.P. (1987a). Human retinoblastoma susceptibility gene: cloning, identification and sequence. *Science* **235**:1394-1399.

Lee, W-H., Shew, J.Y., Hong, F.D., Sery, T.W., donoso, L.A., Young, L-S., Bookstein, R. and Lee, Ey-H.P. (1987b). The retinoblastoma susceptibility gene encodes a nuclear phosphoprotein associated with DNA binding activity. *Nature* **329**:642-645.

Leff, T., Elkaim, R., Goding, C.R., Jalinot, P., Sassone-Corsi, P., Perricaudet, M., Kedinger, C. and Chambon, P. (1984). Individual products of the adenovirus 12S and 13S E1A mRNAs stimulate viral Ella and EIII expression at the transcriptional level. *Proc. Natl. Acad. Sci. USA* **81**:4381-4385.

Lekakh, I.V., Kramerov, D.A., Markusheva, T.V., Ryskov, A.P. and Georgiev, G.P. (1984). Biosynthesis of a small cytoplasmic poly $(A)^+$ RNA, homologous to the repetitve sequence B2 of the mouse genome, is catalyzed by RNA polymerase III. *Doklady Akademii Nauk SSSR Biochemistry* **277**:254-258.

Lemischka, I. and Sharp, P.A. (1982). The sequences of an expressed rat α-tubulin gene and a pseudogene with an inserted repetitive element. *Nature* **300**:330-335.

Leong, K. and Berk, A.J. (1986). Adenovirus early region 1A protein increases the number of template molecules transcribed in cell free extracts. *Proc.Natl.Acad.Sci.* **83**:5844-5848.

Leong, K., Brunet, L. and Berk, A.J. (1988). Factors responsible for the higher transcriptional activity of extracts of adenovirus-infected cells fractionate with the TATA box transcription factor. *Mol. Cell. Biol.* **8**:1765-1774.

Lerner, M.R., Boyle, J.A., Hardin, J.A. and Steitz, J.A. (1981). Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. *Science* **211**:400-402.

Lewis, J.B., Esche, H., Smart, J.E., Stillman, B., Harter, M.L. and Mathews, M.B. (1979). The organization and expression of the left third of the adenovirus genome. *Cold Spring Harbor Symp. Quant. Biol.***44**:493-508.

Lewis, J.B., Fahnestock, M.L., Hardy, M.M. and Anderson, C.W. (1985). Presence in infected cells of non-virion proteins encoded by adenovirus messenger RNAs of the major late transcription regions L0 and L1. *Virology* **143**:452-466.

Lewis, J.B. and Mathews, M.B. (1980). Control of adenovirus early gene expression: a class of immediate early products. *Cell* **21**:303-313.

Lichy, J.H., Field, J., Horwitz, M. and Hurwitz, J. (1982). Separation of the adenovirus terminal protein precursor from its associated DNA polymerase: role of both proteins in the initiation of adenovirus DNA replication. *Proc. Natl. Acad. Sci. USA* **79**:5225-5229.

Lillie, J.W., Green, M. and Green, M.R. (1986). An adenovirus E1A protein region required for transformation and transcriptional repression. *Cell* **46**:1043-1051.

Lillie, J.W. and Green, M.R. (1989). Transcription activation by the adenovirus E1A protein. *Nature* **338**:39-44.

Lillie, J.W., Loewenstein, P.M., Green, M.R. and Green, M. (1987). Functional domains of adenovirus type 5 E1A proteins. *Cell* **50**:1091-1100.

Lin, Y.S. and Green, M.R. (1988). Interaction of a common cellular transcription factor, ATF, with regulatory elements in both E1A and cyclic AMP-inducible promotors. *Proc. Natl. Acad. Sci. USA* **85**:3396-3400.

Linne, T., Jornvall, J. and Philipson, L. (1977). Purification and characterization of the phosphorylated DNA-binding protein from adenovirus-type-2-infected cells. *Eur. J. Biochem.***76**:481-490.

Linzer, D.I.H. and Levine, A.J. (1979). Characterization of a 54kd cellular SV40 tumor antigen present in SV40 transformed and uninfected embryonal carcinoma. *Cell* **17**:43-52.

Linzer, D.I.H. and Nathans, D. (1983). Growth-related changes in specific mRNAs of cultured mouse cells. *Proc. Natl. Acad. Sci. USA* **80**:4271-4275.

Linzer, D.I.H. and Nathans, D. (1984). Nucleotide sequence of a growth related mRNA encoding a member of the prolactin-growth homone family. *Proc. Natl. Acad. Sci. USA* **81**:4255-4259.

Lipp, M., Schilling, R. and Bernhardt, G. (1989). Trans-activation of human MYC: the second promoter is target for the stimulation by adenovirus E1A proteins. *Oncogene* **4**:535-541.

Liu, H.T., Baserga, R. and Mercer, W.E. (1985). Adenovirus type 2 activates cell cyle-dependent genes that are a subset of those activated by serum. *Mol. Cell. Biol.* **5**:2936-2942.

Logan, J., Nicolas, J.D., Topp, W.C., Girard, M., Shenk, T. and Levine, A.J. (1981). Transformation by adenovirus early region 2A temperature-sensitive mutants and their revertants. *Virology* **115**:419-422.

Logan, J., Pilder, S. and Shenk, T. (1984). Functional analysis of adenovirus type 5 early 1B. *Cancer Cells* **2**:527-532.

Lucher, L.A., Symington, J.S. and Green, M. (1986). Biosynthesis and properties of the adenovirus 2 L1-encoded 52,000- and 55,000- Mr proteins. *J. Virol.* **57**:839-847.

Ludlow, J.W., de Caprio, J.A., Huang, C.-M., Lee, W.-H., Paucha, E. and Livingston, D.M. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* **56**:57-65.

Lund, P.K., Moats-Staats, B.M., Simmons, J.G., Hoyt, E., D'Ercole, J., Maratin, F. and van Wyk, J.J. (1985). Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesised as a precursor. *J. Biol. Chem.* **260**:7609-7613.

Lusby, E., Fife, K.H. and Berns, K.I. (1980). Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. *J. Virol.* **34**:402-409.

Lyons, R.H., Ferguson, B.Q. and Rosenberg, M. (1987). Pentapeptide nuclear localization signal in adenovirus E1A. *Mol. Cell. Biol.* **7**:2451-2456.

Maniatis, T., Fritsch, E.F. and Sambrook, J. (Editors) *Molecular cloning: a laboratory manual*, (1982), New York: Cold Spring Harbour Laboratory .

Marcus, C.J., Laughlin, C.A. and Carter, B.J. (1981). Adeno-associated virus RNA transcription *in vivo*. *Eur. J. Biochem.***121**:147-154.

Marcus-Sekura, C.J. and Carter, B.J. (1983). Chromatin-like structure of adeno-associated virus DNA in infected cells. *J. Virol.* **48**:79-87.

Masuda, H., Miller, C., Koeffler, H.P., Battifora, H. and Cline, M.J. (1987). Rearrangement of the p53 gene in human osteogenic sarcomas. *Proc. Natl. Acad. Sci. USA* **84**:7716-7719.

Mathews, M. (1975). Genes for VA-RNA in Ad2. *Cell* **6**:223-229.

Mathews, M.B. (1980). Binding of adenovirus VA RNA to RNA: a possible role in splicing?. *Nature* **285**:575-577.

Matrisian, L.M., Bowden, G.T., Krieg, P., Furstenberger, G., Briand, J.P., Leroy, P. and Breathnach, R. (1986). The mRNA coding for the secreted protease transin is expressed more abundantly in malignant than in benign tumors. *Proc. Natl. Acad. Sci. USA* **83**:9413-9417.

Matrisian, L.M., Glaichenhaus, N., Gesnel, M. and Breathnach, R. (1985). Epidermal growth factor and oncogenes induce transcription of the same cellular mRNA in rat fibroblasts. *The EMBO J.* **4**:1435-1440.

Matsuo, T., Hashimoto, S., Wold, W.S.M., Symington, J., Rankin, A. and Green, M. (1982). Identification of adenovirus 2 early region 4 polypeptides by *in vitro* translation and tryptic peptide map analysis. *J. Virol.* **41**:334-339.

Mayor, H.D, Jamison, R.M., Jordan, L.E. and Melnick, J.L. (1965). Structure and compostion of a small particle prepared from a simian adenovirus. *J. Bacteriol.* **90**:235-242.

McClelland, M., Hamish, J., Nelson, M. and Patel, Y. (1988). KGB: a single buffer for all restriction enzymes. *Nucl. Acids Res.* **16**:365.

McGrogan, M. and Raskas, H.J. (1978). Two regions of the adenovirus 2 genome specify families of late polysomal RNAs containing common sequences. *Proc. Natl. Acad. Sci. USA* **75**:625-629.

McIntyre, P. and Stark, G.R. (1988). A quantitative method of analysing specific DNA sequences directly from whole cells. *Anal. Biochem.* **174**:209-214.

McMaster, G.K. and Carmichael, G.G. (1977). Analysis of single-and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. *Proc. Natl. Acad. Sci. USA* **74**:4835-4838.

McNerney, R., Darling, D. and Johnstone, A. (1987). Differential control of proto-oncogene c-*myc* and c-*fos* expression in lymphocytes and fibroblasts. *Biochem.J.* **245**:605-608.

McPherson, R.A., Rosenthal, L.J. and Rose, J.A. (1985). Human cytomegalovirus completely helps adeno-associated virus replication. *Virology* **147**:217-222.

Mellitis, K.H. and Mathews, M.B. (1988). Effects of mutations in stem and loop regions on the structure and function of adenovirus VA RNA. *The EMBO J.* **7**:2849-2859.

Mercer, W.E., Avignolo, C. and Baserga, R. (1984). Role of the p53 protein in cell proliferation as studied by microinjection of monoclonal antibodies. *Mol.Cell.Biol.* **4**:276-281.

Mercer, W.E. and Baserga, R. (1985). The transformation-related p53 protein: a potential regulator of cell proliferation. *Prog. Clin. Biol. Res.* **177**:171-191.

Mercer, W.E. and Baserga, R. (1988). Expression of the p53 protein during the cell cycle of human peripheral blood lymphocytes. *Exp. Cell. Res.* **160**:31-46.

Mercer, W.E., Nelson, D., DeLeo, A.B., Old, L.J. and Baserga, R. (1982). Microinjection of monoclonal antibody to protein p53 inhibits serum-induced DNA synthesis in 3T3 cells. *Proc. Natl. Acad. Sci. USA* **79**:6309-6312.

Milarski, K.L. and Morimoto, R.I. (1986). Expression of human HSP70 during the synthetic phase of the cell cycle. *Proc. Natl. Acad. Sci. USA* **83**:9517-9521.

Miller, B.W. and Williams, J. (1987). Cellular transformation by adenovirus type 5 is influenced by the viral DNA polymerase. *J. Virol.* **61**:3630-3634.

Miller, S.J., Ricciardi, R., Roberts, B.E., Paterson, B.M. and Mathews, M.B. (1980). Arrangement of messenger RNAs and protein coding sequences in the major late transcription unit of adenovirus 2. *J. Mol. Biol.* **142**:455.

Milner, J. and Cook, A. (1986). Visualisation, by immunocyto-chemistry, of p53 at the plasma membrane of both nontransformed and SV40-transformed cells. *Virology* **150**:265-269.

Milner, J. and Milner, S. (1981). SV40-53K antigen: a possible role for 53K in normal cells. *Virology* **112**:785-788.

Montell, C., Courtois, G., Eng, C. and Berk, A. (1984). Complete transformation by adenovirus 2 requires both E1A proteins. *Cell* **36**:951-961.

Montell, C., Fisher, E.F., Caruthers, M.H. and Bert, A.J. (1982). Resolving the functions of overlapping viral genes by site specific mutagenesis at a mRNA splice site. *Nature* **295**:380-384.

Montminy, M.R., Sevarino, A., Wagner, J.A., Mandel, G. and Goodman, R.H. (1986). Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. *Proc. Natl. Acad. Sci. USA* **83**:6682-6686.

Moore, M., Schaack, J., Baim, S.B., Morimoto, R.I. and Shenk, T. (1987). Induced heat shock mRNAs escape the nucleocytoplasmic transport block in adenovirus-infected HeLa cells. *Mol. Cell. Biol.* 7:4505-4512.

Moran, E. (1988). A region of SV40 large T antigen can substitute for a transforming domain of the adenovirus E1A products. *Nature* **334**:168-170.

Moran, E., Grodzicker, T., Roberts, R.J., Mathews, M.B. and Zerler, B. (1986a). Lytic and transforming functions of individual products of the adenovirus E1A gene. *J. Virol.* **57**:765-775.

Moran, E. and Mathews, M.B. (1987). Multiple functional domains in the adenovirus E1A gene. *Cell* **48**:

Moran, E., Zerler, B., Harrison, T.M. and Mathews, M.B. (1986b). Identification of separate domains in the adenovirus E1A gene for immortalization activity and the activation of virus early genes. *Mol. Cell. Biol.* **6**:3470-3480.

Mowat, M., Cheng, A., Kimura, N., Bernstein, A. and Benchimol, S. (1985). Rearrangement of the cellular p53 gene in erythroleukemic cells transformed by Friend virus. *Nature* **314**:633-636.

Muller, R., Bravo, R., Burchkhardt, J. and Curran, T. (1984). Induction of c-*fos* gene and protein by growth factors precedes activation of c-*myc*. *Nature* **312**:716-720.

Muller, R., Curran, T., Muller, D. and Guilbert, L. (1985). Induction of c-*fos* during myelomonocytic differentiation and macrophage proliferation. *Nature* **314**:546-548.

Murphy, D., Brickell, P.M., Latchman, D.S., Willison, K. and Rigby, P.W.J. (1983). Transcripts regulated during normal embryonic development and oncogenic transformation share a repetitive element. *Cell* **35**:865-871.

Murray, J.D., Bellett, A.J.D., Braithwaite, A.W., Waldron, L.K. and Taylor, I.W. (1982). Altered cell cycle progression and aberrant mitosis in adenovirus-infected rodent cells. *J. Cell.Physiol.* **111**:89-96.

Myers, M.W. and Carter, B.J. (1980). Assembly of adeno-associated virus. *Virology* **102**:71-82.

Nahreini, P. and Srivastava, A. (1989). Rescue and replication of the adeno-associated virus 2 genome in mortal and immortal human cells. *Intervirology* **30**:74-85.

Nakabeppu, Y., Ryder, K. and Nathans, D. (1988). DNA binding activities of three murine jun proteins: stimulation by fos. *Cell* **55**:907-915.

Nakajima, T., Masuda-Murata, M., Hara, E. and Oda, K. (1987). Induction of cell cycle progression by adenovirus E1A gene 13S- and 12S- mRNA products in quiescent rat cells. *Mol. Cell. Biol.* 7:3846-3852.

Navalgund, L.G., Rossana, C., Muench, A.J. and Johnson, L.F. (1980). Cell cycle regulation of thymidylate synthestase gene expression in cultured mouse fibroblasts. *J. Biol. Chem.* **255**:7386-7390.

Nevins, J.R. (1982). Induction of the synthesis of a 70,000 dalton mammalian heat shock protein by the adenovirus E1A gene product. *Cell* **29**:913-919.

Nevins, J.R. and Darnell, J.E. (1978). Groups of adenovirus type 2 mRNAs derived from a large primary transcript: probable nuclear origin and possible common 3' ends. *J. Virol.* **25**:811-823.

Nevins, J.R., Ginsberg, H.S., Blanchard, J.M., Wilson, M.C. and Darnell Jr., J.E. (1979). Regulation of the primary expression of the early adenovirus transcription units. *J. Virol.* **32**:727-733.

Nevins, J.R. and Jenson-Winkler, J.J. (1980). Regulation of early adenovirus transcription: a protein product of early region 2 specifically represses region 4 transcription. *Proc. Natl. Acad. Sci. USA* **77**:1893-1897.

Nishikawa, K. (1983). Assessment of secondary-structure prediction of proteins. Comparison of computerized Chou-Fasman method with others. *Biochim. Biophys. Acta* **748**:285-299.

Ochoa, S. (1983). Regulation of protein synthesis initiation in eukaryotes. *Arch. Biochem. Biophys.* **223**:325-349.

Offringa, R., Smits, A.M.M., Houweling, A., Bos, J.L. and van der Eb, A.J. (1988). Similar effects of adenovirus E1A and glucocorticoid hormones on the expression of the metalloprotease stromelysin. *Nucl. Acids Res.* **16**:10973-10984.

O'Malley, R.P., Duncan, R.F. and Hershey, J.W.B. (1989). Modification of protein synthesis initiation factors and the shut off of host protein synthesis in adenovirus-infected cells. *Virology* **168**:112-118.

Oosterom-dragon, E.A. and Anderson, C.W. (1983). Polypeptide structure and encoding location of the adenovirus serotype 2 late, nonstructural 33k protein. *J. Virol.* **45**:251-263.

Oren, M. and Levine, A.J. (1983). Molecular cloning of a cDNA specific for the murine p53 cellular tumor antigen. *Proc. Natl. Acad. Sci. USA* **80**:56-59.

Oren, M., Maltzman, W. and Levine, A.J. (1981). Post-translational regulation of the 54K cellular tumor antigen in normal and transformed cells. *Mol. Cell. Biol.* **1**:101-110.

Ostrove, J.M., Duckworth, D.H. and Berns, K.I. (1981) Inhibition of adenovirus-transformed cell oncogenicity by adeno-associated virus. *Virol.* **113**:521-533.

Ostrowski, L.E., Finch, J., Krieg, P., Matrisian, L., Patshkan, G., O'Connell, J.F., Phillips, J., Slaga, T.J., Breathnach, R. and Bowden, G.T. (1988). Expression pattern of a gene for a secreted metalloproteinase during late stages of tumor progression. *Mol. Carcin.* **1**:13-19.

Ozkaynak, E., Finley, D., Solomon, M.J. and Varsharsky, A. (1987). The yeast ubiquitin genes: a family of natural gene fusions. *The EMBO J.* **6**:1429-1439.

Paabo, S., Bhat, B.M., Wold, W.S.M. and Peterson, P.A. (1987). A short sequence in the COOH-terminus makes an adenovirus membrane glycoprotein a resident of the endoplasmic reticulum. *Cell* **50**:311-317.

Paabo, S., Nilsson, T. and Peterson, P.A. (1986). Adenoviruses of subgenera B, C, D and E, modulate cell-surface expression of major histocompatibility complex class 1 antigens. *Proc. Natl. Acad. Sci.* **83**:9665-9669.

Page, G.S., Smith, S. and Goodman, H. (1981). DNA sequence of the rat growth hormone gene: location of the 5' terminus of the growth hormone mRNA and identification of an internal transposon-like element. *Nucl. Acids Res.* **9**:2087-2104.

Palmiter, R.D. (1974). Magnesium precipitation of ribonucleo-protein complexes. Expedient techniques for the isolation of undegraded polysomes and messenger ribonucleic acid. *Biochemistry* **13**:3606-3615.

Parada, L.F., Land, H., Weinberg, R.A., Wolf, D. and Rotter, V. (1984). Cooperation between gene encoding p53 tumour antigen and *ras* in cellular transformation. *Nature* **312**:649-651.

Parfett, C.L.J., Hamilton, R.T., Howell, B.W., Edwards, D.R., Nilsen-Hamilton, M. and Denhardt, D. (1985). Characterization of a cDNA clone encoding murine mitogen regulated protein: regulation of mRNA levels in mortal and immortal cell lines. *Mol. Cell. Biol.* **5**:3289-3292.

Parfett, C.L.J., Hofbauer, R., Brudzynski, K., Edwards, D.R. and Denhardt, D.T. (1989). Differential screening of a cDNA library with cDNA probes amplified in a heterologous host: isolation of murine GRP78 (BiP) and other serum-regulated low-abundance mRNAs. *Gene* **82**:291-303.

Parks, W.P., Casazza, A.M., Alcott, J. and Melnick,J.L. (1968). Adeno-associated satellite virus interference with the replication of its helper adenovirus. *J. Exp. Med.***127**:91-108.

Patel, R., Chan, W.L., Kemp, L.M., La Thangue, N.B. and Latchman, D.S. (1986). Isolation of cDNA clones derived from a cellular gene transcriptionally induced by herpes simplex virus. *Nucl. Acids Res.* **14**:5629-5640.

Pei, R. and Berk, A.J. (1989). Multiple transcription factor binding sites mediate adenovirus E1A transactivation. *J. Virol.* **63**:3499-3506.

Pereira, H.G., Huebner, R.J., Ginsberg, H.S. and van der Veen, J. (1963). A short description of the adenovirus group. *Virology* **20**:613-620.

Perricaudet, M., Akusjarri, G., Virtanen, A. and Pettersson, V. (1979). Structure of two spliced mRNAs from the transforming region of human subgroup C adencoviruses. *Nature* **281**:694-696.

Perricaudet, M., Le Moullec, J.M. and Pettersson, U. (1980). The predicted structure of two adenovirus T antigens. *Proc. Natl. Acad. Sci. USA* **77**:3778.

Personn, R.H., Bacchetti, S. and Smiley, J.R. (1985). Cells that constitutively express the herpes simplex virus immediate-early protein ICP4 allow efficient activation of viral delayed-early genes *in trans*. *J. Virol.* **54**:414-421.

Persson, H., Jansson, M. and Philpson, L. (1980). Synthesis and genomic site for an adenovirus type 2 early glycoprotein. *J. Mol. Biol.* **136**:375-394.

Persson, H., Mathisen, B., Philipson, L. and Pettersson,U. (1979). A maturation protein in adenovirus morphogenesis. *Virology* **93**:198-208.

Pettersson, U. Structural and nonstructural adenovirus proteins. In: *The Adenoviruses*, edited by Ginsberg, H.S. (1984), New York: Plenum Press, p. 205-270.

Philipson, L. (1961). Adenovirus assay by the fluorescent cell-counting procedure. *Virology* **15**:263-268.

Philipson, L., Pettersson, U., Lindberg, V., Tibbetts, C., Fennstrom, B. and Person, T. (1975). RNA synthesis and processing in adenovirus-infected cells. *Cold Spring Harbor Symp. Quant.Biol.* **39**:447-456.

Pilder, S., Logan, J. and Shenk, T. (1984). Deletion of the gene encoding the adenovirus 5 early region 1B 21,000 molecular weight polypeptide leads to degradation of viral and host cell DNA. *J.Virol.* **52**:664-671.

Pilder, S., Moore, M., Logan, J. and Shenk, T. (1986). The adenovirus E1B-55K transforming polypeptide modulates transport or cytoplasmic stabilization of viral and host cell mRNAs. *Mol. Cell. Biol.* **6**:470-476.

Pines, J. and Hunter, T. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34 cdc2. *Cell* **58**:833-846.

Pinhasi-Kimhi, O., Michalortiz, D., Ben-Ze'er, A. and Oren, M. (1986). Specific interaction between the p53 cellular tumour antigen and major heat shock protein. *Nature* **320**:182-184.

Prelich, G., Tan, C.-K, Mathews, M.B., So, A.G., Downey, K.M. and Stillman, B. (1987). Functional identity of proliferating cell nuclear antigen and a DNA polymerase-δ auxiliary protein. *Nature* **326**:517-520.

Quinlan, M.P. and Grodzicker, T. (1987). Adenovirus E1A 12S protein induces DNA synthesis and proliferation in primary epithelial cells in both the presence and absence of serum. *J. Virol.* **61**:673-682.

Quinlan, M.P., Sullivan, N. and Grodzicker, T. (1987). Growth factor(s) produced during infection with an adenovirus variant stimulates proliferation of nonestablished epithelial cells. *Proc. Natl. Acad. Sci. USA* **84**:3283-3287.

Redemann, B.E., Mendelson, E. and Carter, B.J. (1989). Adeno-associated virus rep protein synthesis during productive infection. *J. Virol.* **63**:873-882.

Redman, K.L. and Rechsteiner, R. (1989). Identification of the long ubiquitin extension as ribosomal protein S27a. *Nature* **338**:438-440.

Reed, K.E. and Mann, D.A. (1985). Rapid transfer of DNA from agarose gels to nylon membranes. *Nucl. Acids Res.* **13**:7207-7221.

Reich, N.C. and Levine, A.J. (1984). Growth regulation of a cellular tumor antigen, p53 in non-transformed cells. *Nature* **308**:199-201.

Reich, N.C., Oren, M. and Levine, A.J. (1983). Two distinct mechanisms regulate the levels of a cellular tumor antigen, p53. *Mol. Cell. Biol.* **3**:2143-2150.

Reichel, P.A., Merrick, W.C., Siekierka, J. and Mathews, M.B. (1985). Regulation of a protein sythesis initiation factor by adenovirus virus-associated RNA I. *Nature* **313**:196-200.

Reichel, R., Kovesdi, I. and Nevins, J.R. (1988). Activation of a prexisting cellular factor as a basis for adenovirus E1A-mediated transcription control. *Proc. Natl. Acad. Sci. USA* **85**:387-390.

Reichel, R., Neill, S.D., Kovesdi, I., Simon, M.C., Raychaudhuri, P. and Nevins, J.R. (1989). The adenovirus E4 gene, in addition to the E1A gene, is important for trans-activation of E2 transcription and for E2F activation. *J. Virol.* **63**:3643-3650.

Rekosh, D.M.K., Russell, W.C., Bellet, A.J.D. and Robinson, A.J. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell* **11**:283-295.

Renz, M., Verrier, B., Kurz, C. and Muller, R. (1987). Chromatin association and DNA binding properties of the c-*fos* protooncogene product. *Nucl. Acids Res.* **15**:277-292.

Riabowol, K., Draetta, G., Brizuela, L., Vandre, D. and Beach, D. (1989). The cdc2 kinase is a nuclear protein that is essential for mitosis in mammalian cells. *Cell* **57**:393-401.

Rice, S.A. and Klessig, D.F. (1985). Isolation and analysis of adenovirus type 5 mutants containing deletions in the gene encoding the DNA binding protein. *J. Virol.* **56**:767-778.

Rice, S.A., Klessig, D.F. and Williams, J. (1987). Multiple effects of the 72-kDa, adenovirus-specified DNA binding protęin on the efficiency of cellular transformation. *Virology* **156**:366-376.

Richter, J.D., Young, P., Jones, N.C., Krippl, B., Rosenberg, M. and Ferguson, B. (1985). A first exon-encoded domain of E1A sufficient for posttranslational modification, nuclear localization and induction of adenovirus E3 promoter expression in Xenopus oocytes. *Proc. Natl. Acad. Sci. USA* **82**:8434-8438.

Rigby, P.W.J., Diekmann, M., Rhodes, C. and Berg, P. (1977). Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymease I. *J. Mol. Biol.* **113**:237-251.

Roberts, B.E., Miller, J.S., Kimelman, D., Cepko, C.L., Lemischka, I.R. and Mulligan, R.C. (1985). Individual adenovirus type 5 early region 1A gene products elicit distinct alterations of cellular morphology and gene expression. *J. Virol.* **56**:404-413.

Robinson, A.J., Younghusband, H.B. and Bellett, A.J.D. (1973). A circular DNA-complex from adenoviruses. *Virology* **56**:54-69.

Roesler, W.J., van den Bark, G.R. and Hanson, R.W. (1988). Cyclic AMP and the induction of eukaryotic gene transcription. *J. Biol. Chem.* **236**:9063-9066.

Rogel, A., Poplikev, M., Webb, C.G. and Oren, M. (1985). p53 cellular tumor antigen: analysis of mRNA levels in normal adult tissues, embryos and tumors. *Mol. Cell. Biol.* **5**:2851-2855.

Rollins, B.J., Morrison, E.D. and Stiles, C.D. (1988). Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* **85**:3738-3742.

Rollins, B.J., Stiers, P., Ernst, T. and Wong, G.G. (1989). The human homolog of the JE gene encodes a monocyte secretory protein. *Mol. Cell. Biol.* **9**:4687-4695.

Rose, J.A. and Koczot, F. (1972). Adenovirus-associated virus multiplication. Helper requirement for viral deoxyribonucleic acid and ribonucleic acid synthesis. *J. Virol.* **10**:1-8.

Ross, S.R., Levine, A.J., Galos, R.S., Williams, J. and Shenk, T. (1980). Early viral proteins in HeLa cells infected with adenovirus type 5 host range mutants. *Virology* **103**:475-492.

Rotter, V., Abutbul, H. and Ben-Ze'er, A. (1983). p53 transformation-related protein accumulates in the nucleus of transformed fibroblasts in association with the chromatin and is found in the cytoplasm of non-transformed cells. *The EMBO J.* **2**:1041-1047.

Rovinski, B. and Benchimol, S. (1988). Immortalization of rat embryo fibroblasts by the cellular p53 oncogene. *Oncogene* **2**:445-452.

Rovinski, B., Munroe, D., Peacock, J., Mowat, M., Bernstein, A. and Benchimol, S. (1987). Deletion of 5'-coding sequences of the cellular p53 gene in mouse erythroleukemia: a novel mechanism of oncogene regulation. *Mol. Cell. Biol.* **7**:847-853.

Rowe, W.P., Hartley, J.W. and Huebner, R.J. (1956). Additional serotypes of the APC virus group. *Proc. Soc. Exptl. Biol. Med.* **91**:260-262.

Rowe, W.P., Huebner, R.J., Gillmore, L.K., Parrott, R.H. and Ward, T.G. (1953). Isolation of a cytogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc. Soc. Exp. Biol. Med.* **84**:570-573.

Ruley, H.E. (1983). Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture. *Nature* **304**:602-606.

Russell, W.C., Hayashi, K., Sanderson, P.J. and Pereira, H.G. (1967). Adenovirus antigens- a study of their properties and sequential development in infection. *J. Gen. Virol.* **1**:495-507.

Ryskov, A.P., Ivanov, P.L., Kramerov, D.A. and Georgiev, G.P. (1983). Mouse ubiquitous B2 repeat in polysomal and cytoplasmic poly $(A)^+$ RNAs: unidirectional orientation and 3'-end localization. *Nucl. Acids Res.* **11**:6541-6559.

Ryskov, A.P., Ivanov, P.L., Tokarskaya, O.N., Kramerov, D.A., Grigoryan, M.S. and Georgiev, G.P. (1985). Major transcripts containing B1 and B2 repetitive sequences in cytoplasmic poly$(A)^+$ RNA from mouse tissues. *FEBS*.**182**:73-76.

Sadasiv, E.C., Piela, T.H. and Chang, P.W. (1989). Detection of latent avian adenovirus associated virus proteins in chicken cells. *Avian Diseases* **33**:125-133.

Sambrook, J., Botchan, M., Gallimore, P., Ozanne, B., Pettersson, U., Williams, J. and Sharp, P.A. (1975). Viral DNA sequences in cells transformed by simian virus 40, adenovirus type 2 and adenovirus type 5. *Cold spring Harbor Symp. Quant. Biol.* **39**:615-632.

Sambucetti, L. and Curran, T. (1986). The fos protein complex is associated with DNA in isolated nuclei and binds to DNA cellulose. *Science* **234**:1417-1419.

Samulski, R.J. and Shenk, T. (1988). Adenovirus E1B 55-Mr polypeptide facilitates timely cytoplasmic accumulation of adeno-associated virus mRNAs. *J. Virol.* **62**:206-210.

Sandler, A.B. and Ketner, G. (1989). Adenovirus early region 4 is essential for normal stability of late nuclear RNAs. *J. Virol.* **63**:624-630.

Sarma, P.S., Huebner, R.J. and Lane, W.T. (1965). Induction of tumours in hamsters with an avian adenovirus (CELO). *Science* **149**:1108.

Sarma, P.S., Vass, W., Huebner, R.J., Igel, H., Lane, W.T. and Turner, H.C. (1967). Induction of tumours in hamsters with infectious canine hepatitis virus. *Nature* **215**:293-294.

Sarnow, P., Hearing, P., Anderson, C.W., Halbert, D.N., Shenk, T. and Levine, A.J. (1984). Adenovirus early region 1B 58,000 dalton tumour antigen is physically associated with an early region 4 25,000 dalton protein in productively infected cells. *J. Virol.* **49**:692-700.

Sarnow, P., Hearing, P., Anderson, C.W., Reich, N. and Levine, A.J. (1982a). Identification and characterization of an immunologically conserved adenovirus early region 11,000Mr protein and its association wtih the nuclear matrix. *J. Mol. Biol.* **162**:565-583.

Sarnow, P., Ho, Y.S., Williams, J. and Levine, A. (1982b). Adenovirus E1B-58kd tumor antigen and SV40 large tumor antigen are physically associated with the same 54kd cellular protein in transformed cells. *Cell* **28**:387-394.

Sassone-Corsi, P. and Borrelli, E. (1987). Promoter trans-activation of proto-oncogenes c-*fos* and c-*myc*, but not c-Ha-*ras*, by products of adenvirus early region 1A. *Proc. Natl. Acad. Sci. USA* **84**:6430-6433.

Sassone-Corsi, P. (1988). Cyclic AMP induction of early adenovirus promoters involves sequences required for E1A trans-activation. *Proc.Natl.Acad.Sci.* **88**:7192-7196.

Savageau, M.A., Metter, R. and Brockman, W.W. (1983). Statistical significance of partial base-pairing potential: implications for recombination of SV40 DNA in eukaryotic cells. *Nucl. Acids Res.* **11**:6559-6570.

Schick, J., Baczko, K., Fanning, E., Groneberg, J., Burger, H. and Doefler, W. (1976). Intracellular forms of adenovirus DNA: integrated form of adenovirus DNA appears early in productive infection. *Proc. Natl. Acad. Sci. USA* **73**:1043-1047.

Schlehofer, J.R., Ehrbar, M. and zur Hausen, H. (1986). Vaccinia virus, herpes simplex virus and carcinogens induce DNA amplification in a human cell line and support replication of a helpervirus dependent parvovirus. *Virology* **152**:110-117.

Schlesinger, M.J., Ashburner, M. and Tissieres, A. (Eds) *Heat shock- From Bacteria to Man.*, (1982), New York: Cold Spring Harbor Laboratory.

Schmieg, F.I. and Simmons, D.T. (1988). Characterization of the *in vitro* interaction between SV40 T antigen and p53: mapping the p53 binding site. *Virology* **164**:132-140.

Schmitt, R.C., Fahnestock, M.L. and Lewis, J.B. (1987). Differential nuclear localization of the major adenovirus type 2 E1A proteins. *J. Virol.* **61**:247-255.

Schneider, C., King, R.M. and Philipson, L. (1988). Genes specifically expressed at growth arrest of mammalian cells. *Cell* **54**:787-793.

Schneider, R.J. and Shenk, T. (1987). Impact of virus infection on host cell protein synthesis. *Ann. Rev. Biochem.* **56**:317-332.

Schneider, J.F., Fisher, F., Goding, C.R. and Jones, N.C. (1987). Mutational analysis of the adenovirus E1A gene: the role of transcriptional regulation in transformation. *The EMBO J.* **6**:2053-2060.

Schneider, R.J., Weinberger, C. and Shenk, T. (1984). Adenovirus VA1 RNA facilitates the initiation of translation in virus-infected cells. *Cell* **37**:291-298.

Schuermann, M., Newberg, M., Hunter, J.B., Jenuwein, T., Ryseck, R.P., Bravo, R. and Muller, R. (1989). The leucine repeat motif in fos protein mediates complex formation with Jun/AP-1 and is required for transformation. *Cell* **56**:507-516.

Schutzbank, T., Robinson, R., Oren, M. and Levine, A. (1982). SV40 large tumor antigen can regulate some cellular transcripts in a positive fashion. *Cell* **30**:481-490.

Scott, M.R.D., Westphal, K. and Rigby, P.W.J. (1983). Activation of mouse genes in transformed cells. *Cell* **34**:557-567.

Senapathy, P. and Carter, B.J. (1984). Molecular cloning of adeno-associated virus variant genomes and generation of infectious virus by recombination in mammalian cells. *J. Biol. Chem.* **259**:4661-4666.

Senear, A.R. and Lewis, J.B. (1986). Morphological transformation of established rodent cell lines by high level expression of the adenovirus type 2 E1A gene. *Mol. Cell. Biol.* **6**:1253.

Setoyama, C., Frunzio, R., Liau, G., Mudryj, M. and de Crombrugghe, B. (1986). Transcriptional activation encoded by the v-*fos* gene. *Proc. Natl. Acad. Sci. USA* **83**:3213-3217.

Shaw, A.R. and Ziff, E.B. (1980). Transcripts from the adenovirus-2 major late promoter yield a single early family of 3' coterminal mRNAs and 5 late families. *Cell* **22**:905-916.

Shimoja, H. and Yamashita, T. (1968). Induction of DNA synthesis by adenovirus in contact-inhibited hamster cells. *Virology* **36**:422-433.

Shiraki, K., Shimojo, H., Sawada, Y., Vemizu, Y. and Fujinaga, K. (1979). Incomplete transformation of rat cells by a small fragment of adenovirus 12 DNA. *Virology* **95**:127-136.

Shohat, O., Greenberg, M., Reisman, D., Oren, M. and Rotter, V. (1987). Inhibition of cell growth mediated by plasmids encoding p53 anti-sense. *Oncogene* **1**:277-283.

Siegelman, M., Bond, M.W., Gallatin, W.M., St John, T., Smith, H.T., Fried, V.A. and Weissman, I. (1986). Cell surface molecule associated with lymphocyte homing is a ubiquitinated branched-chain glycoprotein. *Science* **231**:823-829.

Simanis, V. and Nurse, P. (1986). The cell cycle control gene $cdc2^{+}$ of fission yeast encodes a protein kinase potentially regulated by phosphorylation. *Cell* **45**:261-268.

Simon, M.C., Kitchener, K., Kao, H.-T., Hickey, E., Weber, L., Voellmy, R., Heintz, N. and Nevins, J.R. (1987). Selective induction of human heat shock gene transcription by the adenovirus E1A gene products, including the 12S E1A product. *Mol. Cell. Biol.* **7**:2884-2890.

Singh, K., Carey, M., Saragosti, S. and Botchan, M. (1985a). Expression of enhanced levels of small RNA polymerase III transcripts encoded by the B2 repeats in simian virus 40-transformed mouse cells. *Nature* **314**:553-556.

Singh, K., Saragosti, S. and Botchan, M. (1985b). Isolation of cellular genes differentially expressed in mouse NIH 3T3 cells and a simian virus 40-transformed derivative: growth-specific expression of VL30 genes. *Mol. Cell. Biol.* **5**:2590-2598.

Slaviceck, J.M., Jones, N.C. and Richter, J.D. (1989). A karyophilic signal sequence in adenovirus type 5 E1A is functional in Xenopus oocytes but not in somatic cells. *J. Virol.* **63**:4047-4050.

Smith, D.H., Velcich, A., Kegler, D. and Ziff, E. (1986). Transcriptional control by the adenovirus type-5 E1A proteins. In: *Cancer Cells and DNA Tumor Viruses: Control of Gene expression and Replication*, edited by Botchan, M., Grodzicker, T. and Sharp, P.A. (1986), New York: Cold Spring Harbor Laboratory pp217-224.

Smith, D.H. and Ziff, E.B. (1988). The amino-terminal region of the adenovirus serotype 5 E1A protein performs two separate functions when expressed in primary baby rat kidney cells. *Mol. Cell. Biol.* **8**:3882-3890.

Sogawa, K., Handa, H., Fujisawa-Sehara, A., Hiromasa, T., Yamane, M. and Fujii-kuriyama, Y. (1989). Repression of cytochrome P-450c gene expression by cotransfection with adenovirus E1A DNA. *Eur. J. Biochem.* **181**:539-544.

Solnick, D. (1981). An adenovirus mutant defective in splicing RNA from early region 1A. *Nature* **291**:508-510.

Solnik, D. and Anderson, M.A. (1982). Transformation-deficient adenovirus mutant defective in expression of region E1A but not region E1B. *J. Virol.* **42**:106-113.

Soprano, K.J., Jonak, G.J., Galanti, N., Floros, J. and Baserga, R. (1981). Identification of an SV40 DNA sequence related to the reactivation of silent rRNA genes in human. mouse hybrid cells. *Virology* **109**:127-136.

Southern, E.M. (1975). Detection of specific sequences among DNA fragments separated by gel electophoresis. *J. Mol. Biol.* **98**:503-517.

Spector, D.J., McGrogan, M. and Rastos, H.J. (1978). Regulation of the appearance of cytoplasmic RNAs from region 1 of the adenovirus 2 genome. *J. Mol. Biol.* **126**:395-414.

Stabel, S., Argos, P. and Philipson, L. (1985). The release of growth arrest by microinjection of adenovirus E1A DNA. *The EMBO J.* **4**:2329-2336.

Staden, R. (1982). An interactive graphics program for comparing and aligning nucleic acid and amino acid sequences. *Nucl. Acids Res.* **10**:2951-2961.

Steeg, P.S., Bevilacqua, G., Kopper, L., Thorgeirsson, V.P., Talmadge, J.E., Liotta, L.A. and Sobel, M.E. (1988a). Evidence for a novel gene associated with low tumor metastatic potential. *J. Natl. Cancer Inst.* **80**:200-204.

Steeg, P.S., Bevilacqua, G., Pozzatti, R., Liotta, A. and Sobel, M.E. (1988b). Altered expression of NM23, a gene associated with low tumor metastatic potential during adenovirus 2 E1A inhibition of experimental metastasis. *Cancer Res.* **48**:6550-6554.

Stefano, J.E. (1984). Purified lupus antigen La recognizes an oligouridylate stretch common to the 3' termini of RNA polymerase III transcripts. *Cell* **36**:145-154.

Stein, R.W. and Whelan, J. (1989). Insulin gene enhancer activity is inhibited by adenovirus 5 E1A gene products. *Mol. Cell. Biol.* **9**:4531-4534.

Stein, R.W. and Ziff, E.B. (1984). HeLa cell ß-tubulin gene transcription is stimulated by adenovirus 5 in parallel with viral early genes by an E1A dependent mechanism. *Mol. Cell. Biol.* **4**:2792-2801.

Stein, R.W. and Ziff, E. (1987). Repression of insulin gene expression by adenovirus type 5 E1A protein. *Mol. Cell. Biol.* **7**:1164-1170.

Steinmeyer, K. and Deppert, W. (1988). DNA binding properties of murine p53. *Oncogene* **3**:501-507.

Stephens, C. and Harlow, E. (1987). Differential splicing yields novel adenovirus 5 E1A mRNAs that encode 30 kd and 35 kd proteins. *The EMBO J.* **6**:2027-2035.

Stillman, B.W., Lewis, J.B., Chow, L.T., Mathews, M.B. and Smart, J.E. (1981). Identification of the gene and mRNA for the adenovirus terminal protein precursor. *Cell* **23**:497-508.

Straus, S.E., Sebring, E.D. and Rose, J.A. (1976). Concatemers of alternating plus and minus strands are intermediates in adenovirus-associated virus DNA synthesis. *Proc. Natl. Acad. Sci. USA* **73**:742-746.

Sturzbecher, H.-W., Chumakov, P., Welch, W.J. and Jenkins, J.R. (1987). Mutant p53 proteins bind hsp 72/73 cellular heat shock-related proteins in SV40-transformed monkey cells. *Oncogene* 1:201-211.

Sugano, S., Stoeckie, M.Y. and Hanafusa, H. (1987). Transformation by Rous saroma virus induces a novel gene with homology to a mitogenic platelet protein. *Cell* **49**:321-328.

Svensson, C. and Akusjarvi, G. (1986). Defective RNA splicing in the absence of adenovirus VA RNAI. *Proc. Natl. Acad. Sci. USA* **83**:4690-4694.

Svensson, C., Pettersson, V. and Akusjarvi, G. (1983). Splicing of adenovirus 2 early region 1A mRNAs is non-sequential. *J. Mol. Biol.* **165**:474-499.

Sweetser, D., Birkenmeier, E., Klisak, I.J., Zollman, S., Sparkes, R.S., Mohandas, T., Lusis, A.J. and Gordon, J.I (1987). The human and rodent intestinal fatty acid binding protein genes: a comparative analysis of their structure, expression, and linkage relationships. *J. Biol. Chem.* **262**:16060-16071.

Swindle, J., Ajioka, J., Eisen, H., Sanwal, B., Jacquemot, C., Browder, Z. and Buck, G. (1988). The genomic organization and transcription of the ubiquitin genes of Trypanosoma cruzi. *The EMBO J.* **7**:1121-1127.

Symington, J.S., Lucher, L.A., Brackmann, K.H., Virtanen, A., Pettersson, V. and Green, M. (1986). Biosynthesis of adenovirus type 2 i-Leader protein. *J. Virol.* **57**:848-856.

T'Ang, A., Wu, K.-J., Hashimoto, T., Liu, W.-Y., Takahoshi, R., Shi, X.-H., Mihara, K., Zhang, F.-H., Chen, Y.Y., Du, C., Qians, J., Lin, Y.-G., Murphree, A.L., Qui, W.-R., Thompson, T., Benedict, W.F. and Fung, Y.-K.T. (1989). Genomic organization of the human retinoblastoma gene. *Oncogene* **4**:401-407.

Taccioli, G.E., Grotewold, E., Aisemberg, G.O. and Judewicz, N.D. (1989). Ubiquitin expression in *Neurospora crassa*: cloning and sequencing of a polyubiquitin gene. *Nucl. Acids Res.* **17**:6153-6165.

Takahashi, M., van Hoosier Jr., G.L. and Trentin, J.J. (1966). Stimulation of DNA synthesis in human and hamster cells by human adenovirus type 12 and 5. *Proc. Soc. Exptl. Biol. Med.* **122**:740-746.

Tattersall, P. and Ward, D.C. (1976). Rolling hairpin model for replication of parvoviruses and linear chromosomal DNA. *Nature* **263**:106-109.

Thimmapaya, B., Weinberger, C., Schneider, R.J. and Shenk, T. (1982). Adenovirus VA1 RNA is required for efficient translation of viral mRNAs at late times after infection. *Cell* **31**:543-551.

Thomas, G.P. and Mathews, M.B. (1980). DNA replication and the early to late transition in adenovirus infection. *Cell* **22**:523-533.

Tigges, M.A. and Raskas, H.J. (1982). Expression of adenovirus-2 early region 4: assignment of the early region 4 polypeptides to their respective mRNAs using *in vitro* translation. *J. Virol.* **44**:907-921.

Tigges, M.A. and Raskas, H.J. (1984). Splice junctions in adenovirus 2 early region 4 mRNA: multiple splice sites produce 18 - 24 RNAs. *J. Virol.* **50**:106-117.

Timmers, H.T.M., de Wit, D., Bos, J.L. and van der Eb, A.J. (1988). E1A products of adenoviruses reduce the expression of cellular proliferation associated genes. *Oncogene Res.* **3**:67-76.

Timmers, H.T.M., van Dam, H., Pronk, G.J., Bos, J.L. and van der Eb, A.J. (1989). Adenovirus E1A represses transcription of the cellular JE gene. *J. Virol.* **63**:1470-1473.

Tokunaga, O., Yaegashi, T., Lowe, J., Dobbs, L. and Padmnabhan, R. (1986). Sequence analysis in the E1 region of adenovirus type 4 DNA. *Virology* **155**:418-433.

Tollefson, A.E., Krajesi, P., Yei,S., Carlin, C.R. and Wold, W.S.M. (1990). A 10,400-molecular-weight membrane protein is coded by region E3 of adenovirus. *J.Virol.* **64**:794-801.

Tollefson, A.E. and Wold, W.S.M. (1988). Identification and gene mapping of a 14,700-molecular-weight protein encoded by region E3 of group C adenoviruses. *J. Virol.* **62**:33-39.

Tratschin, J.-D., Tal, J. and Carter, B.J. (1986). Negative and positive regulation *in trans* of gene expression from adeno-associated virus vectors in mammalian cells by a viral REP gene product. *Mol. Cell. Biol.* **6**:2884-2894.

Tremblay, M.L., Dumont, D.J. and Branton, P.E. (1989). Analysis of phosphorylation sites in the exon 1 region of E1A proteins of human adenovirus type 5. *Virology* **169**:397-407.

Trempe, J.P. and Carter, B.J. (1988a). Alternate mRNA splicing is required for synthesis of adeno-associated virus VP1 capsid protein. *J. Virol.* **62**:3356-3363.

Trempe, J.P. and Carter, B.J. (1988b). Regulation of adeno-associated virus gene expression in 293 cells: control of mRNA abundance and translation. *J. Virol.***62**:68-74.

Trentin, J.J., Van Hoosier, G.L. and Samper, L. (1968). The oncogenicity of human adenoviruses in hamsters. *Proc. Soc. Exp. Biol. Med.* **127**:683-689.

Trentin, J.J., Yabe, Y. and Taylor, G. (1962). The quest for human cancer viruses. *Science* **137**:835-841.

Tsukamoto, A.S., Ponticelli, A., Berk, A.J. and Gaynor, R.B. (1986). Genetic mapping of a major site of phosphorylation in adenovirus type 2 E1A proteins. *J. Virol.* **59**:14-22.

Tyndall, C., Younghusband, H.B. and Bellett, A.J.D. (1978). Some adenovirus DNA is associated with the DNA of permissive cells during productive or restricted growth. *J. Virol.* **25**:1-10.

Ulfendahl, P.J., Linder, S., Kreivi, J.P., Nordquist, K., Sevensson, C., Hultberg, H. and Akusjarvi, G. (1987) A novel adenovirus-2 E1A mRNA encoding a protein with transcription activation properties. *The EMBO J.* **6**:2037-2044.

Van den Elsen, P., Houweling, A. and van der Eb, A. (1983). Expression of region E1B of human adenoviruses in the absence of region E1A is not sufficient for complete transformation. *Virology* **128**:377-390.

Van den Elsen, P.J., de Pater, S., Houweling, A., van der Veer, J. and van der Eb, A.J. (1982). The relationship between region E1A and E1B of human adenoviruses in cell transformation. *Gene* **18**:175-185.

Van der Vliet, P.C. and Sussenbach, J.S. (1975). An adenovirus type 5 gene function required for initiation of viral DNA replication. *Virology* **67**:415-426.

Van Loon, A.E., Rozijn, T.H., de Jong, J.C. and Sussenbach, J.S. (1985). Physiochemical properties of the DNAs of the fastidious adenovirus species 40-41. *Virology* **140**:197-200.

Van Ormondt, H. and Galibert, F. (1984). Nucleotide sequence of adenovirus DNAs. *Curr. Top. Micro. Immunol.* **110**:73-142.

Velcich, A. and Ziff, E. (1985). Adenovirus E1A proteins repress transcription from the SV40 early promoter. *Cell* **40**:705-716.

Verma, I.M. and Sassone-Corsi, P. (1987). Proto-oncogene *fos*: complex but versatile regulation. *Cell* **51**:513-514.

Virtanen, A., Gilardi, P., Naslund, A., Le Moullec, J.M., Pettersson, U. and Perricaudet, M. (1984). mRNAs from human adenovirus 2 early region 4. *J. Virol.* **51**:822-831.

Virtanen, A. and Pettersson, U. (1983). The molecular structure of the 9S mRNA from early region 1A of adenovirus serotype 2. *J. Mol. Biol.* **165**:496-499.

Virtanen, A. and Pettersson, U. (1985). Organization of early region 1B of human adenovirus type 2: Identification of four differentially spliced mRNAs. *J. Virol.* **54**:383-391.

Vogel, S., Rosahl T. and Doerfler, W. (1986). Chromosomal localization of integrated adenovirus DNA in productively infected and in transformed mammalian cells. *Virology* **152**:159-170.

Vrati, S., Mann, D.A. and Reed, K.C. (1987). Alkaline Northern blots: transfer of RNA from agarose gels to Zeta Probe membrane in dilute NaOH. *Mol. Biol. Rep.* **1**:174.

Wahl, G.M., Stern, M. and Stark, G.R. (1979). Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethylpaper and rapid hybridization by using dextran sulfate. *Proc. Natl. Acad. Sci. USA* **76**:3683-3687.

Walton, T.H., Moen Jr., P.T., Fox, E. and Bodnar, J.W. (1989). Interations of minute virus of mice and adenovirus with host nucleoli. *J. Virol.* **63**:3651-3660.

Wang, E.W., Scott, M.D. and Ricciardi, R.P. (1988) An adenovirus mRNA which encodes a 14,700 Mr protein that maps to the last open reading frame of E3 is expressed during infection. *J.Virol.* **62**:1456-1459.

Weber, J., Jelinek, W. and Darnell, J.E. (1977). The definition of a large viral transcription unit late in Ad2 infection of HeLa cells: mapping of nascent RNA molecules labeled in isolated nuclei. *Cell* **10**:612-617.

Webster, K.A., Muscat, G.E.V. and Kedes, L. (1988). Adenovirus E1A products suppress myogenic differentiation and inhibit transcription from muscle-specific promoters. *Nature* **332**:553-556.

Weinberg, D.H. and Ketner, G. (1986). Adenoviral early region 4 is required for efficient viral DNA replication and for late gene expression. *J. Virol.* **57**:833-838.

Weinheimer,S.P. and McKnight,S.L. (1987). Transcriptional and post-transcriptional controls establish the cascade of herpes simplex virus protein synthesis. *J. Mol. Biol.* **195**:819-833.

Weinmann, R., Brendler, T.G., Raskas, H.J. and Roeder, R.G. (1976). Low molecular weight viral RNAs transcribed by RNA polymerase III during adenovirus 2 infection. *Cell* **1**:557-566.

West, M.H. and Bonner, W.M. (1980). Histone 2B can be modified by the attachment of ubiquitin. *Nucl. Acids Res.* **8**:4671-4680.

White, E., Blose, S. and Stillman, B. (1984). Nuclear envelope localization of an adenovirus tumor antigen maintains the integrity of cellular DNA. *Mol. Cell. Biol.* **4**:2865-2875.

White, E. and Cipriani, R. (1989). Specific disruption of intermediate filaments and the nuclear lamina by the 19kDa product of the adenovirus E1B oncogene. *Proc. Natl. Acad. Sci. USA* **86**:9886-9890.

White, E., Spector, D. and Welch, W. (1988). Differential distribution of the adenovirus E1A proteins and colocalization of E1A with the 70-kilodalton cellular heat shock protein in infected cells. *J. Virol.* **62**:4153-4166.

White, E., Denton, A. and Stillman, B. (1988). Role of the adenovirus E1B 19,000-dalton tumor antigen in regulating early gene expression. *J. Virol.* **62**:3445-3454.

Whyte, P., Buchkovich, J., Horowitz, J.M., Friend, S.H., Raybuck, M., Weinberg, R.A. and Harlow, E. (1988). Association between an oncogene and an antioncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. *Nature* **334**:124-129.

Whyte, P., Ruley, H.E. and Harlow, B. (1988). Two regions of the adenovirus early region 1A proteins are required for transformation. *J. Virol.* **62**:257-265.

Whyte, P., Williamson, N.M. and Harlow, E. (1989). Cellular targets for transformation by the adenovirus E1A proteins. *Cell* **56**:67-75.

Wiborg, O., Pederson, M.S., Wind, A., Berlund, L.E., Marcker, K.A. and Vuust, J. (1985). The human ubiquitin multigene family: some genes contain multiple directly repeated ubiquitin coding sequences. *The EMBO J.* **4**:755-759.

Wilbur, W.J. and Lipman, D.S. (1983). Rapid similarity searches of nucleic acid and protein data. *Proc. Natl. Acad. Sci. USA* **80**:726-730.

Wilhelm, S.M., Collier, I.E., Kronberger, A., Eisen, A.Z., Manner, B.L., Grant, G.A., Baker, E.A. and Goldberg, G.I. (1987). Human skin fibroblast stromelysin: structure, glucosylation, substrate specificty and differential expression in normal and tumorigenic cells. *Proc. Natl. Acad. Sci. USA* **84**:6725-6729.

Wilkinson, M. (1988). A rapid and convenient method for isolation of nuclear, cytoplasmic and total cellular RNA. *Nucl. Acid Res.* **16**:10934.

Williams, G.T., McClanahan, T.K. and Morimoto, R.I. (1989). E1A transactivation of the human HSP70 promoter is mediated through the basal transcriptional complex. *Mol. Cell. Biol.* **9**:2574-2587.

Winberg, G. and Shenk, T. (1984). Dissection of overlapping functions within the adenovirus type 5 E1A gene. *The EMBO J.* **3**:1907-1912.

Winocour, E., Callaham, M.F. and Huberman, E. (1988). Perturbation of the cell cycle by adeno-associated virus. *Virology* **167**:393-399.

Wold, W.S.M., Cladaras, C., Magie, S.C. and Yacoub, N. (1984). Mapping a new gene that encodes an 11,600-molecular-weight protein in the E3 transcription unit of adenovirus 2. *J. Virol.* **52**:307-313.

Wold, W.S.M., Cladoras, C., Deutscher, S.L. and Kapoor, A.S. (1985). The 19-KDa glycoprotein coded by region E3 of adenovirus: purification, characterization and structural analysis. *J. Biol. Chem.* **260**:2424-2431.

Wu, B.J., Hurst, H.C., Jones, N.C. and Morimoto, R.I. (1986). The E1A 13S product of adenovirus 5 activates transcription of the cellular human hsp70 gene. *Mol. Cell. Biol.* **6**:2994-2999.

Wu, B.J. and Morimoto, R.I. (1985). Transcription of the human hsp70 gene is induced by serum stimulation. *Proc. Natl. Acad. Sci. USA* **82**:6070-6074.

Wu, L., Rosser, D.S.E., Schmidt, M.C. and Berk, A. (1987). A TATA box implicated in E1A transcriptional activation of a simple adenovirus 2 promotor. *Nature* **326**:512-515.

Yamamoto, M., Maehara, Y., Takahashi, K. and Endo, H. (1983). Cloning of sequences expressed specifically in tumors of rat. *Proc. Natl. Acad. Sci. USA* **80**:7524-7527.

Yancopoulos, G.D., Nisen, P.D., Tesfaye, A., Kohl, N.E., Goldfarb, M.P. and Alt, F.W. (1985). N-*myc* can co-operate with *ras* to transform normal cells in culture. *Proc. Natl. Acad. Sci. USA* **82**:5455-5459.

Yarden, T., Escobedo, J.A., Kuang, W.-J., Yang-Feng, T.L., Daniel, T.O., Tremble, P.M., Chen, E.Y, Ando, M.E., Harkins, R.N., Francke, U., Fried, V.A., Ulrich, A. and Williams, L.T. (1986). Structure of the receptor for platelet-derived growth factor helps define a family of closely related growth factor receptors. *Nature* **323**:226-232.

Yee, S.-P. and Branton, P.E. (1985). Detection of cellular proteins associated with human adenovirus type 5 early 1A polypeptides. *Virology* **147**:142-153.

Yee, S.-P., Rowe, D.T., Tremblay, M.L., McDermott, M. and Branton, P.E. (1983). Identification of human adenovirus early region 1 products by using antisera against synthetic peptides corresponding to the predicted carboxy terminal. *J. Virol.* **46**:1003-1013.

Yeh-Kei, L., Akusjarri, G., Alestrom, P., Pettersson, V., Tremblay, M. and Weber, J. (1983). Genetic identification of an endoproteinase encoded by the adenovirus genome. *J. Mol. Biol.* **167**:217-222.

Yoder, S.S., Robberson, B.L., Leys, E.J., Hook, A.G., Al-Ubaidi, M., Yeung, C.-Y., Kellems, R.E. and Berget, S. (1983). Control of cellular gene expression during adenovirus infection: induction and stimulation of dihydrofolate reductase gene expression by adenovirus type 2. *Mol. Cell. Biol.* **3**:819-828.

Yokota, J., Akiyama, T., Fung, Y.-K T., Benedict, W.F., Namba, Y., Hanaska, M., Wada, M., Terasaki, T., Shimosato, Y., Sugimura, T. and Terada, M. (1988). Altered expression of the retinoblastoma (RB) gene in small-cell carcinoma of the lung. *Oncogene* **3**:471-475.

Yoshinaga, S., Dean, N., Han, M. and Berk, A.J. (1986). Adenovirus stimulation of transcription by RNA polymease III: evidence for an E1A-dependent increase in transcription factor IIIC concentration. *The EMBO J.* **5**:343-354.

Zantema, A., Fransen, J.A.M., Davis-Oliver, A., Ramaekers, F.C.S., Vooijs, G.P., Deleys, B. and van der Eb, A.J. (1985). Localization of the E1B proteins of adenovirus 5 in transformed cells, as revealed by interaction with monoclonal antibodies. *Virology* **142**:44-58.

Zerler, B., Moran, B., Maruyama, K., Moomaw, J., Grodzicker, T. and Ruley, H.E. (1986). Adenovirus E1A coding sequences that enable *ras* and *pmt* oncogenes to transform cultured primary cells. *Mol. Cell. Biol.* **6**:887-899.

Zerler, B., Roberts, R.J., Mathews, M.B. and Moran, E. (1987). Different functional domains of the adenovirus E1A gene are involved in the regulation of host cell cycle products. *Mol. Cell. Biol.* 7:821-829.

Ziff, E.B. and Evans, R.M. (1978). Coincidence of the promoter and capped 5' terminus of RNA from the adenovirus 2 major late transcription unit. *Cell* **15**:1463-1476.

Zimmerman, Jr. J.E., Raska Jr., K. and Strohl, W.A. (1970). The response of BHK 21 cells to infection with type 2 adenovirus. IV activation of DNA-synthesizing apparatus. *Virology* **42**:1147-1150.

Appendix 1

Construction of the subtracted library

The synthesis of a subtractive cDNA library

The subtractive cDNA library was constructed using a modified procedure based on the method described by Hendrick *et al* (1984). Initially it was necessary to synthesise single stranded cDNA from poly A^+ RNA isolated from rat embryo cells prepared 40 hours after infection with WT Ad 5 at a MOI of 25. 10µg poly A^+ cytoplasmic RNA was added to a reaction mix containing 10µl reverse transcription buffer (RTB= 500mM Tris pH8.3, 60mM $MgCl_2$, 600mM NaCl), 10µl 1mg/ml oligo dT primer and 5µl 1mg/ml Actinomycin D. To this was added 2µl each of 100mM dATP, dCTP, dTTP and dGTP , 1µl 1M DTT, 10µl (100µCi) 10mCi/ml ^{32}P dCTP and 10units Reverse transcriptase to a total volume of 100µl. The reaction mix was incubated at 42°C for 60min, 2µl 0.5M EDTA pH7.5 and 20µl 0.5M NaOH were added and further incubated at 70°C for 20min. The solution was neutralised with 10µl 1M Tris HCl pH7.0.

In order to separate the cDNA from unincorporated nucleotides it was necessary to pass the reaction mixture through a 2ml Sephadex G-50 column, pre-equilibrated with NETS buffer (0.1M NaCl, 0.05M Tris-HCl pH7.5,0.001M EDTA and 0.02% SDS). All the fractions collected were subjected to Cerenkov counting and the fractions corresponding to the initial peak of radioactivity were pooled. After the addition of 50µg of bakers yeast tRNA and 1ml of absolute ethanol the sample was stored at -20°C overnight.

The single-stranded cDNA was annealed to a 10 fold excess of poly A^+RNA from dl312 infected cells and a 5 fold excess of sheared denatured viral DNA at 60°C in 0.5M potassium phosphate pH 6.8 (C_0t=2,000 mol s l^{-1}). This involved the ethanol precipitation of the poly A^+ RNA and the cDNA before resuspension in 0.1%SDS, 5mM EDTA, 0.5M potassium phosphate pH6.8 in a total volume of 5µl. The annealing mixture was sealed into a siliconized glass capillary tube and incubated at 65°C for 12hrs.

The single stranded (ss) cDNA molecules were isolated by hydroxylapatite fractionation. Hydroxylapatite was rehydrated in HAP buffer (0.15M sodium phosphate, 0.1% SDS), before application to a 60°C water-jacketed column. The column was equilibrated with 10mls of HAP buffer. The annealing mixture was added to 1ml of HAP buffer at 60°C and applied to the column under slight pressure using a small pump. The eluate, containing the ss cDNA molecules, was collected in 1ml aliquots, using a geiger counter to monitor the progression of radioactivity. HAP buffer was applied to the column until the counts of the eluate aliquots were negligible. Pre-warmed buffer was then added to the clamped column to resuspend the hydroxlapatite, which was then boiled for 5min to dissociate the double stranded material from the column. The hydroxylapatite was

reapplied to the column, and the double stranded solution eluted through the column, as described above.

The ss cDNA sample was concentrated by extraction with an equal volume of 2° butanol until the aqueous layer was reduced to approximately 250µl. Endogenous butanol was removed by extraction with chloroform, followed by an ether extraction.

In preparation for second strand synthesis, the ss cDNA sample was desalted by passing through Sephadex G-50 as described above. 50µg baker's yeast tRNA was added to the pooled eluate representing the ss cDNA and was ethanol precipitated at -20°C overnight.

Second strand cDNA was synthesized by the resuspension of the ss cDNA pellet in 50µl H_2O and incubating at 14°C for 4hrs in the presence of 1mM dNTPs, 5µl α-^{32}P dCTP, 25 units DNA pol 1 Klenow Fragment, 50mM Hepes pH 6.9 (with KOH), 35mM KCl, 5mM $MgCl_2$, in a total volume of 114µl. In order to remove hairpin loops generated by second strand synthesis 400µl of S1 buffer (1mM $ZnSO_4$,30mM sodium acetate, 250mM NaCl) and 400 units Mung bean nuclease was added to the double stranded (ds) cDNA mixture. This was incubated at 37°C for 30mins. The reaction was terminated by the addition of 10µl 0.5M EDTA pH7.5, before extraction with phenol:chloroform (1:1),followed by an ether extraction. The cDNA was ethanol precipitated by addition of 100µl 10M ammonium acetate and two volumes of ethanol before storage overnight at -20°C.

The plasmid vector pUC9 was commercially prepared by cleavage by *Pst1* followed by the enzymatic addition of approximately 12 dG residues to both 3' hydroxy termini. It was necessary to add cytosine nucleotides to the 3'ends of the ds cDNA, in order to ligate to the vector. The tailing reaction comprised of the reprecipitated cDNA, resuspended in 70µl water, 1µl 1mM dCTP, 20µl 5x tailing buffer (=0.5M potassium cacodylate pH7.2, 10mM $CoCl_2$, 1mM DTT), 1µl 10units Terminal deoxynucleotidyl transferase and 5µl 50µCi α-^{32}P dCTP. After incubation at 37°C for 40 mins, the reaction mix was phenol/chloroform extracted three times, followed by two ether extractions. This was followed by ethanol precipitation at -20°C for 90mins. The cDNA was centrifuged, dried under vacuum and resuspended in 100µl TE.

A trial ligation and transformation was attempted, using 5ng tailed ds cDNA and a equimolar amount of pUC9. This was calculated to be 22.5ng, after taking into consideration the size of pUC9 (2.7kb), the average size of cDNA (0.6kb) and the amount of cDNA used ($^{2.7}/_{0.6}$ x 5ng =22.5ng). 5ng of cDNA was added to 22.5ng of pUC9 in a final volume of 27.5µl 1X NTE (0.1M NaCl, 10mM Tris.HCl pH7.8, 1mM

EDTA). This was incubated at 70°C for 3min., before placing into a beaker of 65°C water and allowing to cool to 42°C. The tube was then transfered to a 42°C water bath and incubated for 2 hours. To complete the annealing reaction the mixture was incubated at 14°C for 16hrs and placed at 0°C.

To facilitate the transformation of the recombinant plasmids into bacteria, the annealing reaction was combined with 200µl competent MC1061 *E.coli* and incubated at 0°C for 10 min. This was transferred to 37°C for 45 secs before returning to ice. 700µl of LB was added and the mix was spread onto a L-agar plate containing 10µg/ml Ampicillin. The plate was inverted and incubated at 37°C overnight. The plate yielded approx. 1000 colonies and the trial was deemed sucessful. A ligation was then attempted as described above using the remaining ds material and adjusting the volumes of the other reactants appropriately. 50% of the annealing mix was used to transform MC1061 *E.coli*, as mentioned previously. The transformation mixture was added to 10mls LB, and spread to Amp-L-agar plates at the rate of 1ml per plate. The plates were inverted and incubated at 37°C overnight. Bacteria without transformation mixture and bacteria transformed by unannealed pUC were also plated as controls.

The number of colonies were counted from two plates selected at random. This count enabled the estimation of the number of cDNA clones in the subtracted library. The cDNA library was pooled to facilitate storage and future handling. This was accomplished by adding 10 mls L-broth to each plate and scraping the colonies into the media using a glass spreader. The L-broth was transferred to a sterile 50ml plastic tube and 1ml of sterile glycerol was added before storage at -20°C.

As the subtractive hybridisation technique is not completely efficient it was necessary to screen the library to eliminate plasmids containing common or no cDNA sequences. The pools of bacteria were plated on Amp-L-Agar plates, at a concentration calculated to result in 1000 discrete colonies per plate. The plates were incubated at 37°C until the colonies were distinguishable, and placed at 4°C for 2hrs. Circular nitrocellulose membrane filters were placed on top of the agar plates until the membranes were moist. This was repeated in order to obtain duplicate filters. The orientation of the colonies was marked on the filters before the filters were lifted from the plates and placed (colony side upward) onto 3MM filter paper presoaked in 0.5M NaOH. After 5 min the filters were transferred to filter paper presoaked in 1M Tris-HCl (pH 7.4) for 5 mins. To facilitate binding of nucleic acids the membrane filters were placed on filter papers presoaked in 1M Tris-HCl

pH7.4, 1.5M NaCl. In order to immobilise the DNA, the filters were dried for 1hr at room temperature and baked in vacuo for 2hrs at 80°C.

The nitrocellulose filters were prehybridised, in groups of 5 filters, for 2-3hrs at 68°C in 25mls 5XSSC, 5XDenhardts, 0.1%SDS, 100µg/ml Salmon sperm DNA. Hybridisation mixture was the same as the prehybridisation solution, with the addition of radioactive cDNA probes to a smaller volume (20mls) of solution. Hybridisation proceeded at 68°C for 18-20hrs. Removing unspecific hybridisation involved washing the filters in 2xSSC, 0.1% SDS for 30min at room temp., pre-warmed (to 68°C) 2xSSC, 0.1%SDS for 15min at room temp. twice, and pre-warmed (to 55°C) 1XSSC, 0.1% SDS for 15min at room temp. Filters were exposed to X-ray film at -70°C for 1-4 days.

The autoradiographs were realigned to the original plates and colonies which were differentially expressed, as judged from a comparison between autoradiographs of filters probed with either cDNA from dl312-infected or wt-infected cells, were picked, regrown and rescreened as described above.